Epigenetics in Psychiatry

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Preface

Epigenetics, which literally means above or in addition to genetics, is a neologism that was coined in the 1940s by the British scientist Conrad H. Waddington, combining the words epigenesis (embryonic development) and genetics. Epigenetics has been an active area of research in biomedicine for the past few decades, and in the last decade has become an active area of research in psychiatry.

This book summarizes the findings of research on epigenetics in psychiatry to date. As is evident from many of the chapters of the book, we are presently in the very early stages of this sub-speciality of psychiatry. Although epigenetics holds great promise in illuminating the causes of psychiatric disorders and improving the clinical management of patients with these disorders, there is a long way to go before epigenetics achieves these goals. We hope that this book will help move the subject of epigenetics in psychiatry forward in order for these goals to be achieved.

This book has been written by an international team of experts whom we thank for their excellent contributions. As far as was possible we tried to make sure that there was a common thread running through the book: the correlation of epigenetics in psychiatry with genetics, transcriptomics, and proteomics. The book is comprehensive in its coverage of the subject and is likely to be of interest and use to a wide readership including students and residents in psychiatry, clinical psychiatrists, laboratory researchers, and workers in the pharmaceutical industry dealing with psychotropic drugs. We would like to thank those at Elsevier for their help. In particular we wish to thank Graham Nisbet for his efforts in initiating the writing of the book and Catherine (Cassie) Van Der Laan for her efforts in bringing the book to completion.

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Dr Jacob Peedicayil completed his MBBS in 1984 and MD in pharmacology in 1991, both at the Christian Medical College, Vellore, India. From 1993 to 1995 he did a Post-Doctoral Fellowship at the Centre for Cellular and Molecular Biology, Hyderabad. From 1995 to 1998 he worked as a Research Fellow in the Department of Neurological Sciences, Christian Medical College, Vellore. Since 1998 he has been on the faculty of the Department of Pharmacology and Clinical Pharmacology, Christian Medical College, Vellore, becoming a professor in 2007. He does theoretical research on epigenetics, focusing on epigenetics in psychiatry. In addition, he is involved in experimental research in smooth muscle pharmacology.

Dr Dennis R. Grayson has been interested in mechanisms associated with gene expression for over 35 years. He joined the laboratory of Dr James E. Darnell at the Rockefeller University in 1984 to study cell-type specific transcription factors and their interaction with promoters and enhancers. In 1988, Dr Grayson joined the Fidia-Georgetown Institute for the Neurosciences to study gene expression programs in neurons and continued this research program at Allegheny Singer Research Institute in Pittsburgh from 1994 to 1998. He continued his interests in psychiatry and joined the Psychiatric Institute in 1998. This represented a unique opportunity to pursue the molecular underpinnings of schizophrenia. Dr Grayson has received NRSA post-doctoral support, R01 and K04 funding from the National Institutes of Health to support his work. He has published over 100 papers in peer-reviewed journals and is regularly invited to speak at numerous national and international meetings.

Dr Dimitrios Avramopoulos received his MD from the University of Athens, Greece and his PhD from the University of Crete for his work on mapping human genes on chromosome 21 and deciphering the origin of chromosomal non-disjunction in trisomy 21, mentored by Dr Stylianos Antonarakis. He undertook his post-doctoral work at Johns Hopkins University where he became a faculty member in the Department of Psychiatry in 2002. He is currently on the faculty of the Institute of Genetic Medicine and the Department of Psychiatry at Johns Hopkins University where he works on the genetics of psychiatric disorders. He is interested in the identification of disease causing variants in the genome and the mechanisms through which they affect the brain, individually or in combinations, through the disruption of functional networks and the regulation of the corresponding genes.

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CHAPTER

Introduction to Epigenetics in Psychiatry

1

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CHAPTER OUTLINE

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KEY CONCEPT

- This chapter provides background on the genetics of psychiatric diseases and some of the evidence relating to the importance of epigenetics.

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ABBREVIATIONS

ADHD Attention deficit hyperactivity disorder
ASD Autism spectrum disorder
BP Bipolar disorder
CAR Cortisol awakening response
CNV Copy number variant
FACS Fluorescence-activated cell sorting
GWAS Genome-wide association study
HDAC Histone deacetylase
HPA axis Hypothalamic–pituitary–adrenal axis
LCM Laser-capture microdissection
LOD Logarithm of odds
MDD Major depressive disorder
PGC Psychiatric Genetics Consortium
PTSD Posttraumatic stress disorder
SZ Schizophrenia
TSST Trier Social Stress Test
VCFS Velocardiofacial syndrome
VTA Ventral tegmental area

Introduction

Genetics and epigenetics are closely related concepts. The term epigenetics describes molecular modifications of DNA and histones that act on top of genetic information to regulate gene function. It is therefore impossible to discuss epigenetics independent of genetics and to appreciate the possible roles of epigenetics in psychiatry without a background in the role of genetics in mental illness. The field of psychiatric genetics is relatively new, as is the study of genetics of all complex disorders, primarily because many technological advances were necessary before we could appropriately investigate them. In this chapter, in order to enhance the reader's appreciation and understanding of the important roles that epigenetics can play in shaping the involvement of genes in psychiatric disease, we begin with a quick overview of current knowledge on the genetics of psychiatric disorders, often pointing out places where the relevance of epigenetics seems obvious.

Genetics of psychiatric disorders

The fact that most psychiatric disorders are highly heritable, that a significant fraction of their phenotypic variance in the population is due to genetic factors, has been established for many years and has been the driving force behind the efforts to identify the genes involved. The heritability of a few psychiatric disorders is shown in Table 1.1.

Most psychiatric disorders follow complex inheritance, where despite the clear genetic component the observed patterns cannot fit well into any of the Mendelian modes of inheritance. This is most likely due to the existence of genetic variants in multiple genes that can predispose to disease albeit with a reduced penetrance. There are notable exceptions to this rule, such as early-onset Alzheimer's disease [1], some cases of Parkinson's disease [2], and isolated cases of schizophrenia such as carriers of the velocardiofacial syndrome (VCFS) 22q deletion or the t(1;11) translocation disrupting the *DISC1* gene, where one major genetic defect seems to be involved [3,4]. Although these cases are rare they provide a wealth of information on the biology of disease and as such they are of special interest. Nevertheless, for the vast majority of cases, the genetic etiology remains elusive.

Table 1.1 Heritability of Psychiatric Disorders

Disease	Heritability
Schizophrenia	0.81
Autism spectrum disorder	0.80
Bipolar disorder	0.75
Major depression	0.37
Attention deficit disorder	0.75
Alzheimer's disease	0.58

Source: Sullivan PF, et al. Genetic architectures of psychiatric disorders: the emerging picture and its implications. Nat Rev Genet 2012; 13: 537–52.

The first efforts to identify genes for psychiatric disorders were made in the 1990s, when their heritability was established and the technology for efficient typing of genetic markers was quickly emerging alongside detailed genetic maps of the genome. The hope at the time was that major disease loci were there to be found—loci that would segregate in many of the families and have a relatively high penetrance and loci that could be immediately useful for designing effective treatments. Traditional parametric linkage analysis was initially the method of choice for finding these loci, but it was quickly replaced by non-parametric linkage approaches that were specifically designed for the study of complex diseases. Overall, however, the results were disappointing. Large and laborious studies would result in papers that could report no more than "suggestive" results, and follow-up with larger samples would more often weaken rather than support the original findings. Independent studies on the same disorder would most often not agree on the genomic regions of interest, and even when there was partial agreement it was difficult to prove that it was the result of anything more than chance. At the same time, many researchers were taking the genetic association approach, avoiding questioning the inheritance pattern of genetic variation and simply comparing allele frequencies between cases and controls. A great boost for users of this approach came with discovery of the *APOE* genotype as a risk factor for Alzheimer's disease [5], an association that was strong and held up to almost all replication attempts. It quickly became evident, however, that there was a problem: Small sample sizes gave rise to spurious associations and publication bias to the false impression of replication. Most people in the field were aware that the replications were too rare to be credible, and this was a period of some turmoil within the field of psychiatric genetics.

In 1996, Neil Risch and Kathleen Merikangas published a very influential paper on complex disease genetics [6]. They provided calculations regarding the power of linkage analysis for identifying loci with a range of risk allele frequencies and effect sizes, side by side with the power of association testing. Although Risch later mentioned at a meeting of the American Society of Human Genetics that, on revisiting the calculations, he had found the power of linkage was overestimated, even with the inflated numbers the message was clear: Identifying complex disease loci linkage would require orders-of-magnitude larger samples than those being studied at the time. That paper also showed that genome-wide association studies (GWASs) could identify genes much more efficiently, provided technologies evolved to allow genotyping the number of single nucleotide polymorphisms (SNPs) required to cover the genome. Another decade passed before this last condition was met, but since

then hundreds of new loci have been identified for complex disorders, including some psychiatric diseases. Additionally, the new genotyping arrays have allowed identifying variation in the number of copies of certain genomic regions, deletions, and duplications, together termed copy number variations (CNVs), most of which appear to have no phenotypic consequence but some of which are strongly associated with disease. Below we summarize results of genetic studies for a few psychiatric disorders, beginning with schizophrenia, which has so far provided the most positive results.

Schizophrenia

Schizophrenia (SZ) is one of the best examples of psychiatric disorders in which genetic studies are beginning to bear fruit and the results provide important insights for the study of other disorders. Many GWASs for schizophrenia have been reported, but only recently have we seen the emergence of multiple association signals with strong statistical support similar to the experience with other complex diseases.

The lack of opportunities of early studies led to innovative approaches to extract useful information from the data, including the "risk score" approach [7]. This approach is based on the expectation that among the top signals of a GWAS, even if none reaches the desired levels of significance, one would expect enrichment in true risk variants. The first step is to use the results of a GWAS to calculate effect sizes for the top subset of SNPs. This subset will include both true and false positives. In an independent patient sample, the true positives will tend to have a positive contribution to the score, while the false positives will have random contributions canceling out the contributed scores. Therefore, the calculated score is expected to be higher in individuals carrying more risk alleles (cases) as compared with those that do not (controls). The original study applying this approach [7] not only succeeded in showing its validity but also made an additional important, yet not so unexpected, observation: Risk scores generated from a schizophrenia GWAS were higher not only in cases of SZ but also in cases of bipolar disorder. Although many had proposed this genetic overlap between the two diseases based on linkage and epidemiological studies, this new way to address the question provided very strong new support for the hypothesis and silenced most of the skeptics.

By the time GWASs began revealing loci with good statistical support, study sample sizes had climbed to the tens of thousands, a feat accomplished through collaboration of scientists around the globe in large consortia [8]. In 2011, the Psychiatric Genetics Consortium (PGC) reported on five new SZ loci through a GWAS on a discovery sample of 21,856 samples and a replication sample of 29,839 independent subjects. More recently, after increasing the discovery sample to over 50,000 samples, the PGC announced the identification of over 70 SZ susceptibility loci (XXth World Congress of Psychiatric Genetics, Hamburg, 2012). We are therefore moving into an exciting stage in SZ genetics research where the emphasis will shift toward characterization of the associated loci and identification of the biology underlying the disease. As is the case with other complex disorders, the disease-associated variants explain only a small fraction of the calculated heritability; they are primarily found in non-protein-coding sequences and often there is no obvious gene in the vicinity. Regulatory functions have been shown for some of the non-coding variants, although the majority have not been characterized. Interestingly, among the strongest findings for association with SZ is a microRNA gene that influences the regulation of multiple other genes. It is increasingly clear that gene regulation plays an important role in complex disease risk, which makes the relevance of epigenetics obvious and underlines the importance of the study of epigenetic regulation, the factors that influence it, and the ways in which it can be modified.

The relative risk attributed to sequence variants identified by GWASs is invariably very small, a result that was to be expected given the failures of early studies in gene identification; however, there have been a few examples of very rare variants that show a many-fold increase in risk. The first class of such variants is comprised of CNVs. The first CNV ever shown to be related to SZ was the VCFS region deletion [4]. This ~2-Mb deletion was known to give rise to a distinct syndrome whose phenotype among others included psychosis at a high frequency, and was first shown by Lindsay et al. [9] to be present in a small fraction of SZ patients who did not have a VCFS diagnosis. Follow-up work has confirmed this relationship with no deletions found in controls. Many other CNVs have been found to be associated with SZ with odds ratios that are commonly over 3, and often over 10, indicating high penetrance of the variant. Although such CNVs are extremely valuable in pointing us to genes that require further study, they are very rare and account for only a small fraction of the disease in the population. They are found on many chromosomes (1q21, 3q29, 7q11, 7q36, 15q11, 15q13, 16p13, 16p11, 17p12, and 17q12); they sometimes increase risk in both states, deletion or duplication, and are often involved in more than one psychiatric disorder. A recent paper from Malhotra and Sebat [10] summarizes these events and shows significant overlaps among SZ, bipolar disorder (BP), autism spectrum disorder (ASD), and intellectual disability. Table 1.2 summarizes data reported within a much larger table in that paper, emphasizing the significant overlaps among these diseases.

In addition to inherited common variants and CNVs, *de novo* mutations [11–13] and *de novo* CNVs [14,15] are emerging as a cause of SZ in some cases. A growing number of papers demonstrate this link by showing an increased number of *de novo* mutations in patients and membership of the mutated genes in pathways and networks relevant to SZ [16,17]. The correlation between advanced

Table 1.2 CNVs Showing Effects on Multiple Psychiatric Phenotypes

	SZ	BP	ASD	ID
1a21.1 deletion	8.1 (4.3–15.6)	2.9 (1.2–6.9)	8.0 (3.5–18.4)	12.6 (7.4–21.3)
1q21.1 duplication	4.2 (2.1–8.6)			4.4 (2.6–7.4)
3q29 deletion	63.0 (8.1–491.7)	27.3 (2.5–301.5)	30.0 (1.9–480.4)	41.8 (5.6–311.6)
7q11.23 deletion	—	—	30.7 (3.4–275.1)	++++
7q11.23 duplication				16.5 (2.2–124.5)
VIPR2 (7q36.3) duplication	3.2 (1.5–7.1)	—	—	—
15q11.2 deletion	2.1 (1.6–2.8)	—	42.6 (15.7–115.5)	1.9 (1.6–2.3)
15q11.2 duplication	5.1 (1.4–19.1)	—	10.8 (3.5–33.1)	18.5 (7.1–47.9)
15q13.3 deletion	10.7 (5.4–21.3)	—	—	15.1 (8.4–27.4)
16p13.3 deletion	2.0 (1.1–3.5)	—	9.5 (5.2–17.4)	2.4 (1.8–3.2)
16p11.2 deletion	9.4 (5.3–16.6)	3.9 (1.9–8.2)	11.8 (6.1–22.7)	9.2 (5.8–14.7)
16p11.2 duplication				3.4 (1.8–6.5)
17p12/HNPP deletion	5.7 (2.4–13.7)	—	—	—
17q12 deletion	9.5 (2.4–38.2)	—	16.0 (2.9–87.9)	17.3 (6.1–49.0)
22q11.21 deletion	++++	++++	++++	++++
22q11.21 duplication			3.3 (1.6–6.6)	3.7 (2.3–6.1)

Note: The figures give the odds ratios with 95% confidence intervals.
Abbreviations: CNVs, copy number variants; SZ, schizophrenia; BP, bipolar disorder; ASD, autism spectrum disorder; ID, intellectual disability; ++++, found only in cases.
Source: Data from Malhotra D, Sebat J. CNVs: harbingers of a rare variant revolution in psychiatric genetics. Cell 2012; 148: 1223–41.

paternal age and *de novo* mutation [18] as well as the advanced paternal age observed in SZ [19,20] support these results. *De novo* events by definition do not contribute to heritability and according to the existing reports they only explain a small fraction of cases. It is important to remember, though, that they would not have been linked to SZ except for the very fact that they are *de novo*. Once they enter the population they will often be inherited at least for a few generations, before the natural selection due to the decreased fecundity [21,22] of SZ patients eliminates them, thus contributing to heritability. Although individually rare and undetectable by GWAS, they might be more common collectively, and together with their large effect size contribute significantly to the heritability of SZ. Identifying such variants of higher effect size has the major advantage that they are more likely to produce measurable effects in model organisms and *in vitro* systems, an important step toward their biological characterization.

Finally, other studies that have shown some success in identifying genetic lesions causing schizophrenia are studies of single pedigrees with a heavy load of patients likely to segregate single high penetrance variants. The most successful, perhaps, has been the identification of a Scottish pedigree with a chromosome 1:11 translocation [3] at what is now known as the DISC1 (Disrupted in Schizophrenia 1) locus, which has been extensively followed up mostly by neuroscientists who have been adding to the evidence of its role in psychosis. There are, however, many other examples of such pedigrees [23–26] that, although they showed no chromosomal rearrangement that could immediately identify candidate genes, have provided strong linkage scores. As whole genome sequencing is becoming faster and more cost-effective such pedigrees will likely soon contribute to the identification of additional rare variants of large effect. Recently we proposed a model of disease where the accumulation of multiple hits in gene components of a single biological process is necessary to cause disease, with individual variants having little or no impact [27]. We were able to achieve proof of principle by sequencing genes involved in neuregulin processing. This model, which assumes complex interactions rather than additive effects, is more in line with the widely accepted concepts of robustness and modularity of biological systems and can explain the missing heritability and resistance to purifying selection seen in SZ. Further, it could involve a variety of genetic and environmental hits, some of which could be mediated by reversible epigenetic modifications, an exciting possibility for treatments.

Autism spectrum disorder

Unlike SZ, common variants with robust statistical support have not yet emerged in autism, likely because the sample sizes of GWASs performed so far are smaller, only in the few thousands [28,29]. Nevertheless, it has been shown that common variants are a major source of risk, contributing 40% and 60% of total variation in liability for autism spectrum disorder (ASD) patients from simplex and multiplex families [30], respectively, and it is likely that future larger GWASs will be more fruitful. On the other hand, autism genetics has been very successful in identifying rare disease variants, with some important implications for our understanding of the disease. About 5% of ASD cases, depending on phenotype severity, have been shown to have a visible chromosomal abnormality, whereas 10–15% of individuals have autism as part of a broader identifiable Mendelian condition or genetic syndrome [31,32]. Further, CNVs appear to be a relatively common cause of autism whether they are private *de novo* events, recurrent *de novo* events, or inherited [29]. The CNVs, as well as the identified rare damaging mutations, have led to the emergence of a few specific genes that appear

more consistently and implicate synaptic cell adhesion pathways in the pathogenesis of ASD [33]. Of particular relevance to this book, however, is the involvement of the *MECP2* gene in autism. *De novo* loss-of-function mutations in *MECP2* cause Rett syndrome in approximately 70% of affected females and are lethal in males [29]; however, the phenotype can vary depending on the specific mutation and X-inactivation pattern (*MECP2* is an X-linked gene). Although distinctly different from autism, Rett syndrome often includes autistic features in its manifestations. What's more, *MECP2* mutations have been found in non-syndromic autistic girls [29], and its expression in the frontal cortex has been found to be reduced in the frontal cortex of autistic patients [34]. *MeCP2* binds methylated DNA and, depending on the other protein partners present and the target gene, it can act as an activator or a repressor. This provides a direct link between autism and epigenetic regulation [35,36].

Bipolar disorder

Progress in identifying the genetics of bipolar disorder (BP) is reminiscent of that for SZ; for a recent review, see Craddock and Sklar [37]. GWASs, after initial failures due to small sample sizes, have begun to reveal statistically robust associations. The latest study from the PGC [38] assembled 16,731 samples for discovery and 46,912 for replication and identified two genome-wide significant loci. One was the gene *ODZ4*, whose biology remains poorly understood; the other was *CACNA1C*, a locus that was not new in the literature as it had already been suggested by earlier results. Interestingly, this is also a locus with an association with SZ, which not only adds to its validity but also makes it biologically interesting as a case of genetic overlap. Many other loci summarized in Craddock and Sklar [37] have shown interesting results for BP, including other loci overlapping with SZ. We (unpublished data) and others [39] have found that the BP- and SZ-associated variant in *CACNA1C* is correlated with the transcript levels of the gene. This raises the possibility that, in addition to genetic variation, epigenetic regulation could also be involved in the role of this gene in SZ and BP.

Chromosomal abnormalities, although not as many as in SZ and ASD, have also been associated with BP in the context of broader phenotypes such as 22q11 deletion syndrome (VCFS) [40] and uniparental disomy at 15q11–q13 (Prader–Willi syndrome). The role of CNVs in BP, *de novo* or inherited, appears to be less prominent and is more controversial [14,41,42], which has led to the hypothesis that SZ might require a higher genetic burden, which in turn could be the reason behind its more prominent cognitive dysfunction [42,43]. Nevertheless, some of the same CNVs that have been associated with SZ have also been associated with BP (Table 1.2) [10]. Overall, BP seems somewhat more difficult to untangle than SZ, yet the strong evidence of genetic overlap with SZ is providing significant help toward that end. Further, it supports the idea that, after the age of GWASs, which require large samples often at the expense of phenotyping, detailed clinical characterization of patients will be increasingly important in understanding the relationships among genetics, epigenetics, environment, and phenotypes.

Major depressive disorder

Major depressive disorder (MDD), perhaps not surprisingly, has been estimated to have the lowest heritability of all psychiatric disorders (Table 1.1). Therefore, it may not be surprising that a GWAS of close to 20,000 samples [44] did not reveal any genome-wide significant results, in contrast to studies of other disorders with similar sample sizes. The contribution of structural variation has not

yet been well addressed in the literature, and the evidence that exists shows a relatively moderate excess specifically for deletions with an odds ratio of 1.25–1.52 [45]. Further, MDD was in fact the most common of the phenotypes present in the *DISC1* pedigree, followed by SZ, and it contributed significantly to the linkage raising the logarithm of odds (LOD) score from 3.4 for SZ/BP to 7.1 for SZ/BP/MDD. Given the strong environmental contribution in MDD suggested by the low heritability and the delayed effectiveness of antidepressant medication, it is likely a disorder where epigenetics is particularly relevant, perhaps through the stabilization of regulatory aberrations triggered by the environment and corrected by medications.

Attention deficit hyperactivity disorder

The largest GWAS reported so far for attention deficit hyperactivity disorder (ADHD) included 2064 trios, 896 cases, and 2455 controls, a relatively small sample for this purpose, so the lack of a genome-wide significant result is not surprising. A better picture of the common variant contribution to this disease will arise when larger studies become available. Similar to the other disorders we have discussed, the CNV burden and specific CNVs have been shown to be associated with the disease, including loci previously reported in autism and schizophrenia [46].

Genotype versus phenotype

An interesting observation that emerges from genetic studies of psychiatric disorders, in terms of both common variants with low penetrance and rare inherited or *de novo* variants with higher penetrance, is that the same variants are often involved in multiple disorders. For SZ and BP as well as ASD this was long suspected based on epidemiological studies [47] and linkage studies [48], before being confirmed by GWAS and CNV research as outlined above. In a recent paper [49], the Cross-Disorder Group of the PGC analyzed GWAS data for SZ, BP, MDD, ASD, and ADHD and confirmed that the cross-diagnosis genetic correlations were highest for SZ/BP; moderate for SZ/MDD, BP/MDD, and ADHD/MDD; and low but significant for SZ/ASD. The overlap suggests that some genetic variation compromises the robustness of human mental function in a more general way. An obvious question, then, is what determines the direction that each patient's phenotype will take toward a specific diagnosis and the variations within a given diagnosis? This is sometimes the result of genetic variation, as we and others have shown [50–52], but is also likely due to environmental factors, either directly or through epigenetic modifications. An obvious long-term goal for medical research is to understand each of these factors for each patient so treatment can be targeted where it will provide the most effective and desirable modification of the phenotype for mental health and well-being.

Missing heritability

As we discussed, GWASs have succeeded in providing the first common variants and pointing to genes relevant to mental health as they have done for many complex disorders. Although this trend is expected to expand to more psychiatric diagnoses and accelerate, one thing is becoming increasingly clear: The additive effects of common variants identified by GWASs will not account for most

of the heritability calculated by genetic studies. Although this does not in the least diminish the value of the identified variants and genes, it does raise the question of the source of this missing heritability. Many answers have been proposed, including rare variants and CNVs but also inflated estimates of the calculated heritability due to non-additive genetic effects (gene–gene interactions), shared family environments, and gene–environment correlations and interactions [53].

The possible involvement of rare single nucleotide variants is now beginning to be addressed, as exome and genome sequencing technologies have made it possible to sequence and compare multiple genomes. Many difficulties remain, however. The low frequency is detrimental to statistical power unless the effect size is quite large. It is unknown how many rare variants of modest effects there might be and therefore how much they might contribute to heritability. The problem of power is exacerbated by the great number of rare variants identified by sequencing, as we move from correcting for one to several million statistical tests. Reducing their number by filtering variants for functional significance is a valid approach, but our filters are currently not adequate, either for coding variants or even more so for non-coding variants. Grouping variants into genes—for example, exploring the "mutational burden" of each gene—could be useful but such an approach suffers from many limitations, as unfortunately most of the variants included in such summations are likely to be irrelevant to gene function and some might have opposite effects on the risk. Until our tools for more accurate prediction of the functional consequences of variants improve, these problems will remain significant obstacles in identifying rare disease variants.

There are two main approaches to uncovering rare single nucleotide variants. The first is the exploration of *de novo* mutations, an approach inspired somewhat by the observation of *de novo* CNVs which has already provided some promising results as mentioned earlier. It is likely that the complexity of the nervous system and the biology involved in mental processing provide a large target for new mutations to hit. It is always challenging to prove that a given *de novo* variant is the cause of disease, and it is not unlikely that it results in disease only in individuals with additional genetic and environmental risk factors. Nevertheless, current studies provide compelling evidence of a causal link. Although *de novo* variants do not contribute to heritability, they do point to specific genes and disease-causing defects that can be of extreme value. The second approach is the study of large families with high disease burden. Some such families might carry high-penetrance disease variants that can be identified by genome sequencing and followed by linkage analysis to show their relationship to the disease. Although some of the same limitations apply, such as the lack of concrete functional annotation on DNA variation and extreme rarity of the variants, this approach has already proven effective for other incompletely penetrant disorders [54] and has led to the identification of high-penetrance variants that as we argued above can be of increased value for research.

A significant fraction of heritability is likely to remain unexplained, hidden in rare variants with small effects, at least until datasets become much larger, perhaps not until every individual born is sequenced and systematically followed up. Technological advances make this sound less and less like science fiction, although there are many, especially legal, issues that will have to be addressed before the world engages in such policies. Although identifying most rare variants would be of interest, there remain some unexplored hypotheses regarding where the missing heritability could be attributed. As we mentioned earlier, whereas the calculated narrow-sense heritability refers to additive genetic effects, non-additive effects as well as gene–environment correlations and interactions can inflate this calculation [53,55]. In fact, non-additive effects could be predicted to be more common than additive effects, as genes work in interactive biological pathways and biological pathways are known to

be robust, resistant to change as a result of individual genetic changes. Robustness allows for larger pools of potentially functional variation, which in turn are advantageous and expected to promote evolvability [56], a possibility particularly relevant for the rapidly evolved primate brain. In contrast, unless positively selected through pleiotropic effects, variation with additive effects is vulnerable to purifying selection and likely to disappear. In recent work from our lab exploring the genetics of schizophrenia, we showed that this is likely the case and that with innovative approaches to sequencing data analysis one is likely to capture such interactions [27]. It is therefore likely that, aided by new knowledge gained from GWASs as well as the progress made in neuroscience with regard to characterizing the biological processes active in the brain, we will soon be able to obtain a much better understanding of the genetics of psychiatric disease.

Another little explored source of heritability is intrafamilial correlations due to epigenetic modifications driven by parental exposures. It has been shown that modifications of the epigenome can persist for a few generations [57]. Both genetic and environmental factors can lead to such modifications, and their persistence can generate familial phenotype correlations that are not due to transmitted genetic variation. Regardless of whether this fits the definition of heritability or not, it would be measured as such and would not be detected by current genetic approaches. What is more, knowing how such variation influences the phenotype could lead to epigenetics-based approaches to treatment.

Epigenetics in psychiatric disorders

While psychiatric genetics continues to gain momentum with improvements in sample size, statistical power, and new sequencing technologies, the field of psychiatric epigenetics has also begun to gain traction in the past several years. Studies examining epigenetic mechanisms of psychiatric disease complement ongoing efforts in genetics by providing a functional context and the means to quantify non-genetic elements and environmental factors contributing to disease. Emerging epidemiological and clinical evidence has begun to uncover that maternal malnutrition, immune activation, and early-life adversity are a few of many environmental factors that can influence risk to psychiatric illnesses. The effects of these factors could be through epigenetic modifications interacting with genetic variation to precipitate disease. Despite the high heritability of the majority of the psychiatric disorders (Table 1.1), there is ample space for epigenetics to play a significant role, and under certain circumstances it might inflate this calculated heritability. And, even more so than genetic sequence, epigenetic marks are more amenable to modification, providing the possibility of altering or reversing the consequences of exposure to disease-causing environmental factors through medications or manipulation of the epigenetic machinery.

In the second half of this introductory chapter, we provide epidemiological and clinical evidence for the involvement of epigenetics in psychiatric illnesses, including a discussion of environmental factors, visit a few recent case studies that have implemented innovative techniques, and demonstrate important principles and concepts for epigenetics research. We bring attention to the need to establish an epigenetic framework for psychiatric illnesses through bodies of information and refinements in techniques at basic science, preclinical, and clinical levels. As the field of epigenetics is growing, there is a need to standardize methods and approaches so data can be meta-analyzed and unified, as has been done for psychiatric genetics.

Although concordant phenotypes from twin studies have been used to support the high heritability and the need for the study of genetics for a majority of psychiatric illnesses, discordance provides a

unique opportunity to investigate novel disease mechanisms. For instance, the discordant phenotype of monozygotic twins for major depression is presumably due to non-genetic factors, likely including epigenetic mediators that affect brain physiology, and can be a useful approach to identifying disease loci [58]. This may be especially relevant for disorders such as depression, where heritability is about half that of bipolar disorder or schizophrenia (Table 1.1). In fact, several key studies have examined discordant epigenetic signatures between monozygotic siblings as a source of non-genetic disease burden, including studies on BP [59], SZ [60], MDD [58], and ASD [61].

An important clue implicating epigenetic factors comes from the mechanism of action of mood stabilizers and antidepressants. There is ample evidence that many medications alter epigenetic modifications to influence gene function. This raises the possibility that the epigenetic structure supporting the risk and protective alleles or the expression of a gene whose levels are important for disease may be an important modifier of their contribution to the risk. The considerable time required to achieve therapeutic response is consistent with the need for epigenetic modifications to be altered for protective transcripts to be robustly enhanced or risk alleles suppressed. Although expression changes are likely relevant even in the absence of disease-associated DNA variation, this possibility represents an interesting link between genetics and epigenetics. We now know of several medications impacting epigenetic alterations by affecting key enzymes that modify DNA or histones. For example, it has been known for over a decade that valproic acid, or the bipolar drug Depakote, is a histone deacetylase (HDAC) inhibitor [62]. Its effect on acetylation of histone H3 is achieved by inhibiting expression of several HDACs, primarily those in Class I and II. Similar effect has been observed with the tricyclic antidepressant, imipramine, on *Bdnf* and HDAC5 [63]. Other histone modifications have been implicated in drug addiction. It has recently been shown that cocaine-induced plasticity is mediated by the histone methyltransferase G9a [64]. Together, these findings suggest that in addition to disease vulnerability contributed by genetic variations, the epigenetic structure surrounding disease-relevant genes may also play a role.

Epidemiological and clinical studies of psychiatric diseases are replete with several environmental factors as notable risk factors for psychiatric disease. Whether through interactions with genetic variations or in their own right, environmental factors can cause persistent changes to gene function when they target specific signal transduction pathways and culminate in epigenetic alterations. For example, caloric restriction and infections that occur during pregnancy are associated with schizophrenia, giving rise to the idea that these factors interfere with or impede normal fetal and postnatal neurodevelopment. Factors such as stress, trauma, and early-life adversity have been associated with anxiety and mood disorders such as posttraumatic stress disorder (PTSD), MDD, and BP, with these disorders being more closely associated with a perturbation of the hypothalamic–pituitary–aderenal axis (HPA axis), which governs the stress response and regulates homeostatic glucocorticoid levels.

Nutrition

One of the more striking epidemiological studies linking nutrition or caloric restriction and psychiatric diseases (in this case, SZ) examined the Dutch famine winter of 1944 to 1945. Susser et al. [65] assessed the risk for SZ in individuals whose mothers were exposed to severe caloric restriction during the first trimester and found a twofold increase in individuals conceived at the peak of the famine. Interestingly, this observation was replicated in another cohort, this time during the Great Famine

in China from 1959 to 1961, and again demonstrated approximately twice the risk for SZ [66]. The possibility that epigenetic modifications to the mother or child caused by famine mediate an increase in risk for disease is intriguing, yet very little work has been reported that explores it. An epigenetic study in the Dutch cohort observed a famine-associated decrease in blood DNA methylation at a key regulatory region of the insulin-like growth factor 2 (IGF2) gene [67], a gene that has been demonstrated to be important for brain development. However, given the relatively small difference in DNA methylation (<6%) and the absence of an established correlation between DNA methylation in blood and the brain for *IGF2*, this matter requires further investigation. Evidence of diet causing epigenetic changes to the offspring *in utero* comes from work on the A^{vy} allele in the Agouti mouse, where prenatal exposure to a diet rich in one-carbon nutrients, such as methionine, folate, and choline, causes a drastic change in the pups from yellow to mosaic brown coat color via an increase in DNA methylation of a transposon in the promoter of the A^{vy} gene [68]. Although animal behavior was not investigated in this classical study, other studies report DNA methylation-associated changes in dopamine and opioid systems of pups exposed pre- and postnatally to a high-fat diet [69,70]. These studies are likely to have significant relevance to diet-induced epigenetic and behavioral changes in humans.

Prenatal infection

An abundance of studies have examined the relationship between gestational exposure to infections and SZ. Epidemiological studies consisting of epidemic outbreaks and birth cohort studies have linked exposure to influenza, usually in women whose mothers were infected during the first trimester, with increased risk for SZ [71]. A recent study also observed a fourfold increase in the risk of BP after maternal exposure to influenza during pregnancy [72]. Similar associations have been made with other pathogens such as herpes simplex virus type 2 [73] and more notably with *Toxoplasma gondii* [74]. Toxoplasmosis is known to have potent teratogenic consequences on the developing fetus. Maternal inflammatory and immune responses provoked by infections may also play a role in psychiatric diseases. In fact, many studies have linked elevated levels of specific cytokines during pregnancy, such as interleukin-8 (IL-8) and tumor necrosis factor α (TNFα), with increased incidence of SZ [73,75]. Behavioral studies of animals that were prenatally exposed to maternal cytokines or elevated immune system via polyI:C administration during pregnancy support the clinical studies [76,77]. An interesting example of gene–environment interaction can be found in Abazyan et al. [78], who reported that prenatal exposure of mice harboring a dominant negative allele of the human *DISC1* to poly I:C was accompanied by behavioral deficits not observed in untreated transgenic or treated wild-type animals. Despite the promising data, studies of diet and infection-induced psychiatric diseases do not establish clear relationships among the environmental factor, epigenetics, and behavior. Moreover, many of these factors account for relatively minor differences in odds ratios, no larger than most genetic factors, and like them, when the sample sizes are small, are prone to nonreplication by independent groups. This is a field of study that clearly must grow in order to answer these intriguing questions.

Stress

Exposure to stress is one of the strongest and best-studied environmental factors for anxiety and mood disorders. The role of environmental factors may be especially relevant to mood and anxiety

disorders, given that these disorders have a much lower heritability (Table 1.1). Preclinical [79,80], epidemiological [81], and clinical studies [82] suggest a strong link between exposure to stress and dysregulation of the HPA axis or susceptibility to neuropsychiatric illnesses. Many studies support the notion that exposure to stress in its various forms, duration, and intensity all burden the HPA axis and the glucocorticoid dynamics it regulates [83]. Specifically, perception and experience of the stressor by the individual activates the HPA axis, whose neuroendocrine endpoint is the production and release of the human glucocorticoid cortisol. It is thought that both intense traumatic stress and chronic stress disrupt the homeostatic and tight negative-feedback regulation of the HPA axis, ultimately leading to abnormal cortisol levels that persist even in the absence of the stressor. For example, clinical studies of PTSD or early childhood trauma have documented persistent alterations in baseline cortisol measurements such as the cortisol awakening response (CAR) [84,85] or dysregulation of cortisol response during a challenge of the HPA axis by the dexamethasone (DEX) suppression test (and CRH/DEX) [86–88] and the Trier Social Stress Test (TSST) [89]. These findings suggest that in addition to tissue-specific processes that are transcriptionally influenced by cortisol signaling, genes that directly regulate and mediate the stress response of the HPA axis, and subsequent cortisol levels, are also targets of cortisol action.

This intimate relationship between stress or cortisol-induced HPA axis dysregulation and further perturbation of cortisol dynamics is thought to have a profound consequence on mood and emotions. A disease that exemplifies this relationship is Cushing's syndrome, where hypercortisolemia due to adrenocorticotropic hormone (ACTH)-secreting pituitary adenomas is associated with failure to suppress endogenous cortisol during the dexamethasone suppression test and a notable 60–90% comorbidity with MDD [90–92]. Alleviation of Cushing's syndrome patients' depressive symptoms with resolution of hypercortisolemia by removal of the offending tumors suggests a causal role for excessive cortisol and HPA axis dysregulation in MDD [91,93]. Similarly, a landmark epidemiological study has followed hundreds of thousands of patients who were administered glucocorticoids (i.e., iatrogenic Cushing's) for a wide variety of non-psychiatric systemic disorders and found a significant increase in cases of MDD, suicide, mania, and anxiety associated with glucocorticoid therapy [94].

Glucocorticoids, one of the primary agents of stress response in chordates, directly influence DNA methylation and chromatin remodeling [95,96]. The approaches and methodologies employed in studying stress provide excellent examples of epigenetic studies that can be applied to other environmental factors. We will briefly review two case studies, one in animals and one in humans, that capitalize on some of the mentioned advantages (below) and exemplify innovative techniques in epigenetics. Although some "classical" works have examined the effects of social stress [63] and early-life environment [97] on gene function, here we focus on more recent developments.

Niwa et al. [98] imposed social isolation stress on adolescent mutant *DISC1* transgenic mice that resulted in hypermethylation of the promoter of the tyrosine hydroxylase (*Th*) gene, an HPA axis target gene implicated in psychosis. Specifically, the *Th* promoter became densely methylated in dopaminergic neurons of the ventral tegmental area (VTA) that project into the mesocortical layer, whereas those that project into the mesolimbic regions did not. Further, isolation-induced epigenetic and behavioral deficits became mitigated in the presence of the glucocorticoid receptor (GR) antagonist mifepristone (RU486), directly implicating glucocorticoid signaling in epigenetic control of behavior. What is notable about this study is that it implements many of the principles requisite in epigenetic studies. First, it demonstrates the importance of crosstalk between genetics and epigenetics. The more severe consequences of dopamine insufficiency observed in the transgenic mice suggest

a role for genetics in exacerbating the impact of social stress. In humans, it is likely to be the case that many of the psychiatric diseases arise as a result of contribution by both genetic and epigenetic factors. Second, the study demonstrates an innovative use of cellular enrichment techniques to examine a very small population of cells in the brain. Identification and enrichment for disease-relevant cellular units is a prerequisite to observe appreciable epigenetic differences in the brain. The use of retrograde beads to label neurons that project into different brain regions, combined with fluorescence-activated cell sorting to isolate them, has allowed the authors to identify epigenetic differences within dopaminergic neurons of the VTA that are not only stress specific but also projection specific. Third, by using the GR antagonist RU486 to reverse the constellation of symptoms associated with dopaminergic insufficiency, Niwa et al. [98] clearly implicated glucocorticoid signaling.

Another study by Klengel et al. [99], this time in human lymphocytes, reported early childhood trauma-induced loss of methylation on a blood-specific, intronic region of the *FKBP5* gene. Glucocorticoid- and trauma-induced changes in DNA methylation and expression of *FKBP5* are consistent with glucocorticoid resistance and hypercortisolemia. In this study, a crucial SNP (rs1360780) implicated in several independent studies of mood and anxiety disorders had a moderating effect on DNA methylation and expression, mediating a gene–environment interaction [99]. What is remarkable in this study is the authors' ability to uncover functional significance of the SNP using what is referred to as chromosome-conformation capture, a technique that can be used to assess long-range interactions between regulatory elements and the promoter and that has significant application in epigenetics. By showing increased interactions between the intronic region harboring the SNP and the promoter, the authors demonstrated mechanistically how a non-exonic variant can affect gene function. Others have shown similar interactions within the GR gene [100]. In addition, given that the study is in humans, the authors also demonstrated a significant correlation between the epigenetic changes and the severity of trauma exposure determined through psychometric assessments.

Epigenetic versus genetic approaches to psychiatric disorders

Despite the overlaps in the types of experiments and approaches used by genetic and epigenetic studies, there are also significant differences that often make epigenetic approaches more challenging. Genome-wide or whole-genome studies that have identified disease-associated SNPs and their epigenomic counterparts require extensive statistical scrutiny to derive genome-wide significance and avoid false-positive findings. Further, any of the "hits" that are identified through genetic and epigenetic screens have to be characterized through rigorous molecular biology in terms of their influence on gene function. In GWASs, exonic SNPs must be evaluated in terms of their consequence on protein structure and function, whereas those residing in intergenic or intronic regions must be evaluated by their influence on transcription. For regions associated with differential DNA methylation or histone modifications, their functional significance is derived from their effect on transcription factor binding and chromatin structure, respectively, although effects on alternative splicing and transcription initiation are also increasingly recognized as a potential mechanism [101]. These similarities arise as a result of the common endpoint, which is alteration in gene function.

A critical difference is that unlike genetic information, which is mostly static or "set in stone," epigenetic information is dynamic and governed by context, through space (tissue specificity) and

time (developmental process). Whereas the nucleotide sequence of DNA is essentially identical no matter when or where the sampling occurred (with notable exceptions such as in tumors), epigenetic marks are not. Methylation of DNA and the plethora of covalent modifications on histones vary depending on types of tissue and during time of development. This spatial and temporal context poses a considerable obstacle and challenge to epigenetic studies because specific disease-affected tissues must be isolated during a specific time in development in order for the disease-relevant changes to be detected. For neuropsychiatric diseases, this problem is especially relevant given that their primary target is mostly inaccessible in live humans. Even in animal models this is exceptionally challenging, as the heterogeneous nature of brain tissues serves as a significant confounding factor. The need to isolate cell types, such as neurons from glia, in order to increase the observable effect size in DNA methylation was demonstrated by a postmortem study on suicide by Labonte et al. [102]. There are a number of methods available to circumvent this problem. Specific targeting of the paraventricular nucleus as described by Elliott et al. [103], isolation of neuronal nuclei by fluorescence-activated cell sorting (FACS) [102], microdissection of specific neurons by laser-capture microdissection (LCM), and the use of retrograde beads followed by FACS to isolate projection-specific neurons of VTA as described in Niwa et al. [98] are a few notable examples.

Another difference, this time an advantage of epigenetics research, is the increased benefit derived from animal models. Whereas genetic studies have employed gene knockouts or the introduction of human alleles in mouse models, such as *DISC1* and *BDNF* [98,104], there are significant limitations in the interpretation of their function in such a different genetic context. For certain types of studies, however, animals are very well suited for the study of psychiatric epigenetics, although not without limitations; for a review on model validity, see Nestler and Hyman [105]. Environmental factors such as caloric restriction, immune activation, and exposure to medications and stressors all employ similar physiological processes and signal pathways among mammals. Perhaps the biggest advantage of using animal models to study the impact of environmental factors on physiological processes is the availability and accessibility of brain tissue. As such, a great amount of insight has been gained by animal research that would not have been possible through postmortem or brain imaging studies in humans. Animal studies allow control over many parameters that cannot be controlled in humans, such as drug exposure, lifestyle, and time of tissue collection, among others.

Although animal models cannot fully recapitulate all of the nuances of human mental conditions, they remain indispensable in our understanding of epigenetic consequences of environmental risk factors. Unbiased epigenomic screens consisting of either DNA methylation platforms or chromatin immunoprecipitation methods of neuronal populations during a specific developmental period and environmental exposure will be necessary to identify disease-relevant gene sets and pathways and cannot be as systematically performed in humans. The study of behavior and rescue of phenotype by pathway- or protein-specific drugs will strengthen the significance of the screen findings and will provide a strong rationale to study the role of identified genes in behavior. Knockout and transgenic animals of genes selected through such genome-wide screens or allelic variations implicated in human studies can be generated to assess the role of these genes in affecting related behaviors. For example, genes such as *Crh*, *Gr*, *Fkbp5*, *Bdnf*, and *Disc1* have been altered in animals to show their involvement in stress-induced behaviors [98,103,106–108]. Experiments that involve gene manipulations have the power to assign causal relationships to observations that otherwise can be associative at best.

Moving epigenetics forward

In order for the field of psychiatric epigenetics to move forward, it must be grounded on a foundation of established and standardized observations and methodologies. Additional tools and more comprehensive experiments are necessary to establish the role of epigenetics in psychiatry and the way epigenetics complements and interacts with genetics. Some of the studies and technical refinements necessary to advance our understanding of epigenetics in psychiatric disorders are summarized in Table 1.3.

Human studies aimed at developing treatments will undoubtedly be facilitated and accelerated if supported by strong research across complementary fields. Animal models can be used to establish correlative relationships between brain and peripheral tissues that can be immensely useful for biomarker discovery. Tools for site-specific manipulation of the epigenome can be used in animal models to observe the role of DNA methylation and histone modification in gene regulation *in vivo*. One of the great hopes of epigenetics research is the possibility of site-specifically altering epigenetic structure. Recent advances in zinc finger targeting of specific enzymatic activities may provide the means of achieving this goal [109]. Basic science experiments elucidating the mechanism of action of environmental risk factors are also necessary. One of the first tasks of such experiments is to identify specific methyltransferase and demethylase activities for DNA and histones and how they interact with the epigenome. For stress and glucocorticoids, some of the epigenetic enzymes have been implicated, including DNMT1 and HDAC5 [63,110]. It is much less clear what mechanism is employed by other environmental factors such as nutrition. In addition, it is important to identify complexes of proteins and transcription factors that coordinate the physiological processes in response to environmental factors. Identification of these proteins will broaden our understanding of the epigenetic machinery and allow for additional targets of more efficacious drug intervention.

Human epigenetic studies have the inherent limitations of tissue accessibility for live subjects and specific tissue availability for postmortem specimens. Foundational knowledge established by basic science research and animal models can be used to strengthen human studies conducted in clinical settings or on postmortem brain tissues. Epigenomic loci in blood, which is one of the most easily accessible

Table 1.3 Some Studies and Technical Refinements Required to Advance Our Understanding of Epigenetics in Mental Disorders

Type of Study	Efforts Needed
Basic research	• Characterization of epigenetic enzymes and pathways • Functional characterization of locus-specific epigenetic changes • Improvements in the enrichment of homogeneous cellular populations
Translational research	• Transgenic and knockout models • Site-specific manipulation of the epigenome • Effects of drugs and environment on the epigenome
Research on humans	• Epigenomic studies in postmortem brains • Correlation of epigenetic effects between tissues • Identification of peripheral biomarkers of psychiatric disorders • Integration between genetics and epigenetics
Research strategies	• Establish norms to facilitate the integration of datasets across studies

tissues, should be validated as proxies for those in brain that emerge from studies in animals. Causal relationships between alterations of target genes and behavioral deficits derived from animal models can provide a stronger argument for a causal role of specific genes in diseases. For example, recent findings of epigenetic differences in postmortem brain tissues of suicide completers and controls are supported by studies that have implicated the same genes with aggression and anxiety in animals [102]. It is likely that similar benefits can be obtained to augment imaging studies to hone in on specific regions and circuitry implicated in animal studies. One of the key tenets of epigenetics is the concept of change. As such, development of epigenetic medications that target specific enzymatic processes or even specific epigenomic targets to *reverse disease phenotype* should be pursued.

Conclusion

These are exciting times for those studying the biology of psychiatric disease. As the genetic underpinnings of disease are beginning to emerge and new technologies are promising more breakthroughs in genetics, the same is happening in epigenetics, allowing a better understanding of the dynamic nature of the genome, beginning *in utero* and continuing throughout life. Figure 1.1 is an attempt to summarize the complex interactions between the genome and the environment. It is important to observe the circularity, as genes determine phenotypes (including disease status), phenotypes determine behaviors, behaviors modify some environmental exposures, and the latter can modify epigenetic marks and the function of genes. This is particularly relevant in psychiatry where behavior is directly relevant to disease. Understanding these relationships and breaking such self-feeding circles would be key to our battle against psychiatric disease.

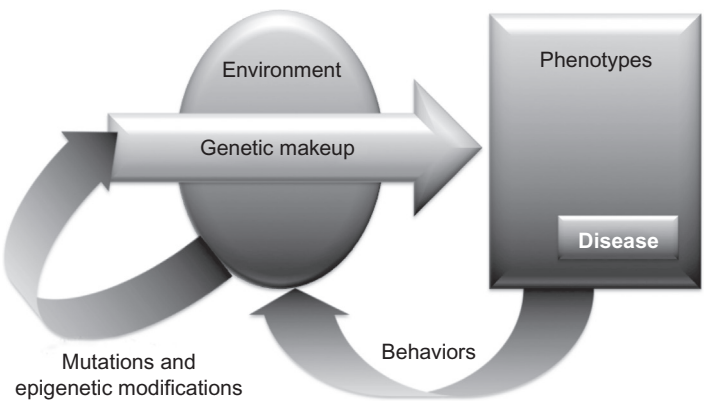

FIGURE 1.1

Our genetic makeup interacts with our environment to give rise to our general phenotype which can include disease. Our phenotype determines our behaviors and our own modifications to our environment. The environment we live in will alter our genes, either through mutation or, more commonly, through epigenetic modification. This dynamic cycle leads to the continuously changing phenotype through aging and can determine whether or not a disease state is reached.

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Glossary

Genetic association A statistical approach to identifying loci that increase the risk for disease. It tests whether the allele frequencies of a polymorphic marker are different in cases than in controls. A positive result is assumed to mean that the allele more frequent in cases is increasing the risk for disease or is tagging another variant allele that does. Today, it is most often applied at the genome-wide level, involving millions of markers and requiring very robust statistical significance to avoid type 1 errors.

Heritability The portion of the variance of a trait in a population that can be attributed to genetics. Narrow-sense heritability, h^2, is the portion of the variance that is explained by additive genetic effects; broad-sense heritability, H^2, is the fraction explained by all genetic effects including interactions.

Linkage analysis An analytical process that explores whether or not two variant sequences segregate independently in pedigrees. When one of the variants is an assumed unobserved disease locus then linkage is said to be between DNA markers and a disease. The relationship between the unobserved genotype at the disease locus and the phenotype is the linkage model. In non-parametric linkage, a model does not need to be specified and the analysis is exploring whether in pairs of relatives ascertained to be concordant for the disease there is on average greater sharing of alleles identical by descent than expected for the given relationship at any location in the genome.

CHAPTER

Outline of Epigenetics

2

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KEY CONCEPTS

- Epigenetics includes processes such as DNA methylation, histone modifications, chromatin remodeling, and effects of non-coding RNA.
- Epigenetics plays an important role in the pathogenesis of various diseases.
- Epigenetic therapy has tremendous potential for the prevention and treatment of disease.

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ABBREVIATIONS

5-aza-CR 5-azacytidine
5-aza-CdR 5-aza-2′-deoxycytidine
5mC 5-Methylcytosine
AP-2 Activating protein 2
ASOs Antisense oligonucleotides
CBP CREB-binding protein
ChIP Chromatin immunoprecipitation assay
CpG Cytosine–phosphate–guanine
CREB cAMP response element-binding protein
DNMT DNA methyltransferase
ESC Embryonic stem cell
GNAT GCN5 *N*-acetyltransferase
HAT Histone acetyltransferase
HDAC Histone deacetylase
Hmc Hydroxymethyl cytosine
HMT Histone methyltransferase
iPSCs Induced pluripotent stem cells
lncRNA Long non-coding RNA
MBD Methyl-CpG-binding domain
MeCP Methyl-CpG-binding protein
miRNA microRNA
MS Mass spectrometry
MYST MOZ, YBF2, SAS2, TIP60
ncRNA Non-coding RNA
NF-κB Nuclear factor κ-light-chain-enhancer of activated B cells
PCAF p300/CBP-associated factor
piRNA PIWI-interacting RNA
PRMT Protein arginine methyltransferase
RTS Rubinstein–Taybi syndrome
siRNA Small interfering RNA
SMA Spinal muscular atrophy
SMN Survival motor neuron gene
snoRNA Small nucleolar RNA

Introduction

With the completion of the Human Genome Project, there was a huge fervor among geneticists, who were of the belief that our genes determine our destiny. However, even though the vast majority of cells in a multicellular organism share an identical genotype, the phenotype may be distinct. During cellular differentiation and growth, distinct epigenetic patterns govern gene expression. These epigenetic patterns are what Waddington described as the "epigenetic landscape" [1]. Arthur Riggs and colleagues have precisely defined epigenetics as the study of mitotically and meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence [2]. A number of exciting findings revealed that nucleotide sequences are not the only determining factors. Certain chemical alterations on the nucleotides govern which genes are expressed and when they are expressed. A big question is how the genes are selectively expressed. Genes must be accurately repressed and activated at the appropriate time for the proper functioning of the organism. Several diseases such as cancer and neurodevelopmental disorders occur because of the inappropriate repression or activation of genes. Some classic epigenetic modifications include DNA methylation, posttranslational modifications of histone proteins, silencing of the extra copy of the X chromosome in females, and genomic imprinting. In addition, protein complexes such as sumo proteins and polycomb group proteins have been reported to have epigenetic-modifying properties. Recently, non-coding RNA (ncRNA) such as microRNA (miRNA), small interfering RNA (siRNA), and long non-coding RNA (lncRNA) have generated considerable interest due to their role in several biological processes and disease pathogenesis. They have also been included under the umbrella definition of epigenetics because of their involvement in several epigenetic processes; however, there is some question as to whether non-coding RNA is truly heritable and epigenetic.

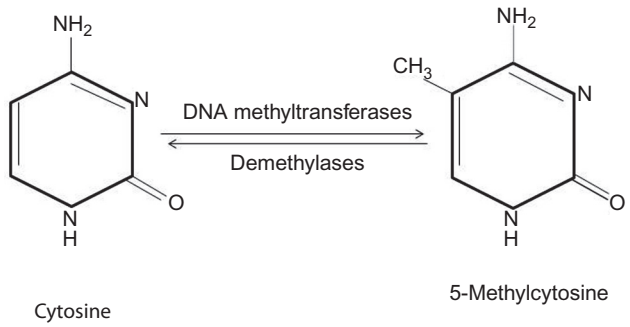
Molecular mechanisms of epigenetics

DNA methylation

DNA methylation is crucial for development. DNA methylation patterns are established early during embryogenesis and maintained throughout adulthood. Alterations of these established patterns can lead to gene silencing and a multitude of diseases; therefore, it is not surprising that DNA methylation is one of the most important and studied of all epigenetic processes. DNA methylation occurs by the addition of a methyl group to the carbon 5 position of the cytosine pyrimidine ring predominantly in the cytosine–phosphate–guanine dinucleotide-rich regions (see Figure 2.1).

Cytosine–phosphate–guanine (CpG) islands are found in the 5′ regulatory regions of most human genes and are generally in a non-methylated state except for the CpG islands on the inactive X chromosome in females [3]. These islands comprise about 1% of the genome. In stable normal cells, repetitive DNA sequences are often methylated. CpG islands also have a markedly open chromatin structure that is deficient in the linker histone H1 and contain nucleosomes enriched in acetylated forms of histones H3 and H4 [4]. There are several proposed mechanisms for the repression of transcription by DNA methylation. One mechanism involves the physical impedance of transcription factors binding to a certain region of the gene due to methylation of promoter regions. Methylation inhibits the binding of several transcription factors such as AP-2, c-Myc/Myn, the cyclicAMP-dependent activator *CREB*, E2F, and NF-κB. These transcription factors recognize sequences that contain CpG residues but cannot bind to methylated residues within their recognition sequence [5,6]. Another mechanism involves recruitment of methyl-CpG-binding domain (MBD) proteins. This is an indirect mechanism and involves repressors such as MeCP2, MBD1, MBD2, MBD4, and Kaiso [7].

The enzymes involved in DNA methylation are DNA methyltransferases (DNMTs) (see Figure 2.2). The most prominent enzymes of the DNMT family are DNMT1, DNMT3a, and DNMT3b. DNMT1 is the most abundant methyltransferase in somatic cells [8]. Methylation can be *de novo* or maintenance.



Conversion of cytosine to 5-methylcytosine by DNA methyltransferases

FIGURE 2.1

Conversion of cytosine to 5-methylcytosine. DNA methyltransferases adds a methyl group to the carbon 5 position of the pyrimidine ring. This is a reversible process, and demethylases remove the methylated cytosines from DNA.

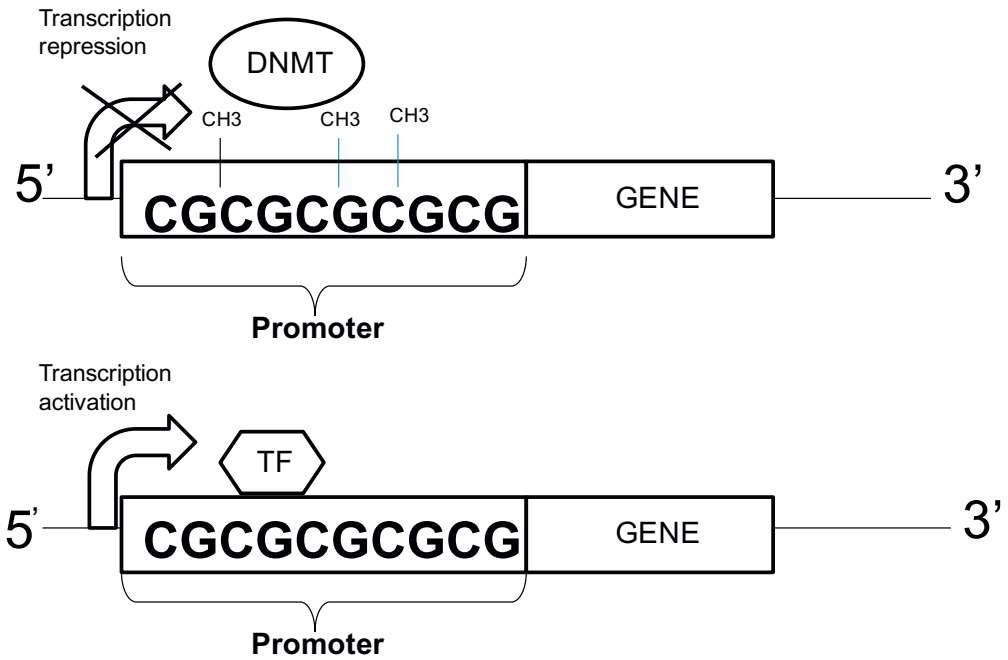


FIGURE 2.2

DNA methyltransferases (DNMTs) bind to the promoter region of genes and catalyze methylation of CpG. Methyl residues prevent the binding of transcription factors to DNA and generally lead to transcription repression.

In *de novo* methylation, CpG dinucleotides on both the strands are non-methylated when DNA methylation occurs, whereas maintenance refers to copying methylation patterns onto a hemimethylated parental template after DNA replication [9]. DNMT1 primarily has maintenance methyltransferase activity but it also has minor *de novo* methylating activity and is required for proper embryonic development, imprinting, and X inactivation [10–12].

The DNMT3 family of genes is known to be highly conserved, as similar genes have been found in zebrafish, *Arabidopsis thaliana*, and maize [13]. DNMT3a and DNMT3b are powerful *de novo* methyltransferases, and recently they have been shown to play an important role in initiation and proliferation of stem cells [14]. Another group of DNA methylation-modifying enzymes, referred to as demethylases, are also important as they remove the methylated cytosines from DNA, thus disrupting DNA methylation patterns that were established early during embryogenesis. MBD2b has shown demethylating properties, and 5-methylcytosine glycosylase removes the methylated cytosine from DNA, leaving the deoxyribose intact [15]. Another group of proteins that act as demethylating agents are the ten-eleven translocation (TET) proteins, which catalyze the conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC); hmc is not recognized by MeCP2 or DNMT1. DNMTs are generally overexpressed in cancer. DNMT1 and DNMT3b are known to be highly cooperative, as inactivation of both DNMT1 and DNMT3b in HCT116 human colon carcinoma cells results in very

low methylation [16]. Inactivation of both DNMT3a and DNMT3b disrupts *de novo* methylation of proviral DNA in embryonic stem cells (ESs) and genome-wide *de novo* methylation occurring during early development [17].

DNA methylation, development, and disease

DNA methylation is important for normal embryonic development. During early development, innumerable changes occur in the methylation of the mammalian genome that plays a vital role in the development of various tissues and organs. At fertilization, the paternal genome undergoes DNA demethylation. During preimplantation development, genome-wide demethylation occurs followed by global remethylation after implantation [18]. These events are very important for the perfect initiation of embryonic gene expression. Each cell type establishes its own DNA methylation pattern, which is more or less stably maintained throughout life, although various environmental and other factors can change epigenetic patterns. Recent studies have revealed that diet plays a very important role in establishing and changing DNA methylation patterns throughout the life of an individual [19]. Aging is also an important factor and can induce various changes in the body including epigenetic patterns. In general, genomic DNA methylation tends to decrease with aging [16,20].

Properly established and maintained DNA methylation patterns are essential for mammalian development and for the normal functioning of the adult organism. DNA methylation is a potent mechanism for silencing gene expression and maintaining genome stability in the vast quantity of repetitive DNA, which can otherwise mediate recombination events and cause transcriptional deregulation of nearby genes [21]. Several diseases are known to occur because of defects in DNA methylation. The most extensively studied are DNA methylation defects in cancer. Gene-specific hypermethylation is one of the major causes of oncogenesis. Despite prevalent increases in DNA methylation of specific genes, overall deficiencies in the 5-methylcytosine content of DNA are found in almost every type of cancer. Ovarian epithelial carcinomas, prostate metastatic tumors, leukocytes from B-cell chronic lymphocytic leukemia, hepatocellular carcinomas, cervical cancer, high-grade dysplastic cervical lesions, and colon adenocarcinomas have all shown cases of global hypomethylation.

In addition, several nervous system disorders have been linked to DNA methylation. Spinal muscular atrophy (SMA), a common neuromuscular disorder, is caused by the homozygous absence of survival motor neuron gene 1 (SMN1), while the disease severity is mainly influenced by the number of survival motor neuron gene 2 (SMN2) copies [22]. The comprehensive analysis of SMN2 methylation in patients suffering from severe, compared with mild, SMA carrying identical SMN2 copy numbers revealed a correlation of CpG methylation at the positions −290 and −296 with disease severity and the activity of the first transcriptional start site of SMN2 at position −296. The importance of DNA methylation in the brain has been demonstrated by its association with some neurological disorders such as Rett syndrome and immunodeficiency, centromere instability, and facial anomalies (ICF) syndrome. Rett syndrome, which is the most common cause of mental retardation in females, is caused by mutations of methyl-CpG-binding protein 2 (MeCP2) [23]. Mutations of the DNMT3B gene have been reported in patients with ICF syndrome [23]. DNA methyl-CpG-binding proteins are important for proper functioning of the brain and stability of neural cells. Mutant MBD1 mice display decreased hippocampal neurogenesis and impaired spatial learning. Thus, methylation plays a significant role in higher neural functioning and memory formation [24].

Chromatin remodeling and histone modifications

DNA is wrapped around spools known as histones, which are octameric proteinaceous structures. The core histones are comprised of four proteins known as H2A, H2B, H3, and H4. In histones, 147 base pairs of DNA are wrapped 1.65 times around the octamer structure and are stabilized with the assistance of linker histones known as H1. This organization is collectively referred to as chromatin. There is a very strong ionic bond between the negatively charged DNA phosphate backbone and the highly positively charged amino acids of the nucleosomes. This tight and compact structure inhibits the binding of other proteins to DNA. Not all of the DNA is bound to histones, and between every pair of nucleosomes, along the length of chromosomal DNA, are gaps referred to as linker DNA. This DNA is much more accessible to proteins and transcription factors.

There are two ways in which chromatin can be organized. Euchromatin is relatively loosely packed and hence the transcriptionally active form of organization. Heterochromatin, on the other hand, is the compact and transcriptionally inactive form of organization. The N-terminal tails of each histone protrude out and are sites for various covalent posttranslational modifications such as methylation, acetylation, phosphorylation, ubiquitination, sumoylation, and ADP ribosylation. Histone modifications can also occur in the globular domain of histones. There are two ways in which histone modifications regulate transcription. First, the modifications change the structure and orientation of the chromatin; as a result, they become more accessible to transcription factors. The other mechanism is via signals. The first modification often acts as a signal for subsequent histone modifications by recruiting specific enzymes.

Histone acetylation

Histone acetylation refers to the addition of acetyl groups to lysine residues of histones. Two enzymes are involved in the process: histone acetyltransferases (HATs) and histone deacetylases (HDACs) (see Figure 2.3). HATs transfer an acetyl group from acetyl-CoA to lysine residues and are of two types: HAT-A and HAT-B. Type A HATs acetylate nucleosomal and chromatin-associated proteins, whereas type B HATs acetylate cytoplasmic histones that have been newly synthesized [25,26]. Acetylation is primarily associated with transcriptional activation, and most of the acetylation sites fall within the N-terminal tail of the histones. Acetylation of histones makes the nucleosomal DNA more accessible to transcription factors. Three main families of HATs transfer acetyl groups to lysine residues of the nucleosome core histones: the MOZ, YBF2, SAS2, TIP60 (MYST) family; the GCN5 *N*-acetyltransferase (GNAT) family; and the CREB CBP/p300 family [27]. HATs such as p300, CBP, and p300/CBP-associated factor (PCAF) acetylate multiple non-histone proteins, many of which play prominent roles in oncogenesis [28]. The p300/CBP is a transcriptional coactivator protein. It plays a very important role in regulating transcription, and it impacts a multitude of cellular processes such as cell-cycle control, differentiation, and apoptosis [29].

Histone deacetylases (HDACs) are opposite in function to HATs as they remove the acetyl moiety from lysine residues. They are of three types: Class I (HDAC1, 2, 3, 8), Class II (HDAC4, 5, 6, 7, 9), and Class III based on their similarity to yeast proteins. Class III HDACs require NAD+ for their activity and are called sirtuins. They are seven in number (SIRT1–7). Histone chaperones identify the acetylation pattern on the histone and then assist this assembly on the nucleosomes [29]. Once the synthesis of histone is completed in the *S*-phase of the cell cycle, histones may be rapidly modified by HATs, which catalyze the transfer of an acetyl moiety from acetyl coenzyme A onto the ε-amino group of lysine residues.

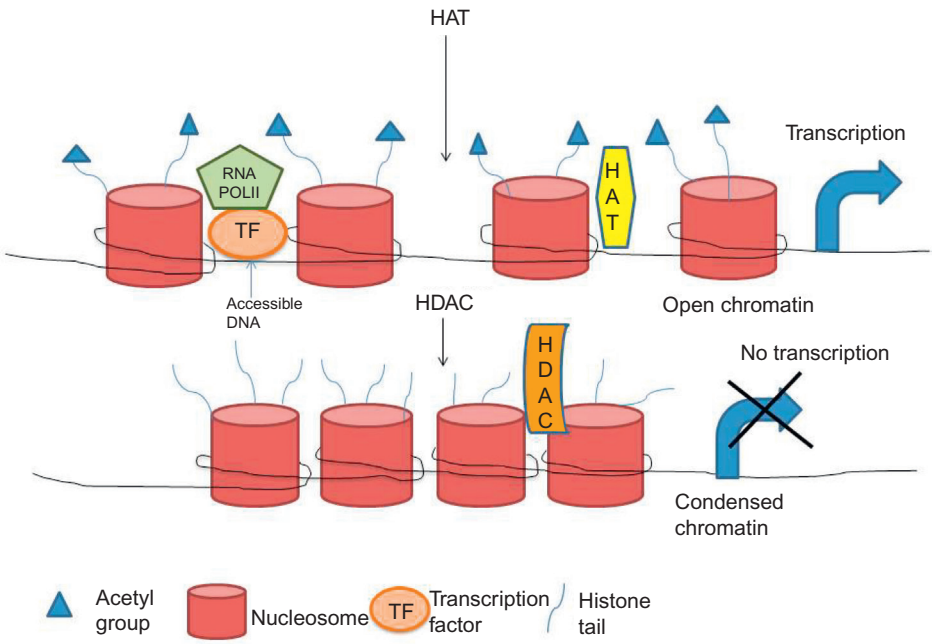


FIGURE 2.3

Acetylation of histones by histone acetyltransferase (HAT) generally leads to an open chromatin structure; thus, transcription factors and RNA polymerase can bind to DNA and activate transcription. Histone deacetylases (HDACs) deacetylate histone and lead to a closed chromatin state where the transcription factors cannot bind.

Both targeted and global acetylation/deacetylation affect the level of gene transcription. Research in the past few decades has suggested that acetylation stimulates gene transcription, whereas deacetylation represses transcription. Recent data, however, indicate that the situation is more complex because histone deacetylation is also a key factor in ensuring proper transcription [30].

Both HAT and HDAC enzymes generally are part of multiprotein complexes. There are numerous examples in which subunits of HAT or HDAC complexes influence their substrate specificity and lysine preference, which in turn affect the broader functions of these enzymes. In addition, HATs and HDACs also regulate processes such as DNA recombination, replication, and repair, but the precise functions of acetylation in these and other processes have yet to be elucidated [31].

Aberration of HAT causes a variety of diseases. Mutations in the human CBP gene have been reported to be associated with Rubinstein–Taybi syndrome (RTS), which is a developmental disorder with an increased risk of cancer development [32]. The p300/CBP is a global transcriptional coactivator that plays a critical role in a variety of cellular processes, including cell-cycle control, differentiation, and apoptosis. A recent study suggests that decreases in CBP and acetylated histone levels lead to a subsequent decrease in HAT activity and may represent a common pathway in various neuropathologies [33]. TIP60 is a HAT of the MYST family that may play an important role in regulating tumorigenesis, apoptosis, and DNA repair [34]. Depletion of TIP60 prevents p53 from inducing expression of p21 [35].

Several reports have shown that acetylation of the C-terminal regulatory domain is involved in regulating activity of p53. Acetylation of this site is observed after DNA damage *in vivo* which may induce p53 and cause cell-cycle arrest or apoptosis; therefore, overexpression of PCAF can cause growth arrest [36]. The adenoviral E1A oncoprotein represses transcriptional signaling by binding to p300/CBP and displacing PCAF and p/CIP proteins from the complex. E1A directly represses the HAT activity of both p300/CBP and PCAF *in vitro* and p300-dependent transcription *in vivo*. Additionally, E1A inhibits nucleosomal histone modifications by the PCAF complex and blocks p53 acetylation [37].

Histone methylation

Lysine and arginine residues can be methylated by enzymes termed histone methyltransferases (HMTs). Lysine can be mono-, di-, or triacetylated, whereas arginine can be monomethylated, symmetrically dimethylated, or asymmetrically dimethylated. The HMTs contain a conserved SET domain. Arginine methylation is catalyzed by a family of protein arginine methyltransferases (PRMTs), and both HMTs and PRMTs are very specific with respect to the residue that is methylated. In contrast to histone acetylation, histone methylation is a relatively long-lived mark. Methylation of histones is effective in recruiting certain transcription factors to chromatin. There is a strong association between the methylated histones and DNA and/or chromatin. Some protein domains that can recognize and bind to specific methylated lysines are chromodomains, tudor domains, PhD fingers, and WD40 repeat domains [38]. Methylation does not alter the overall charge of the histones. Methylation of H3K9, H3K27, and H4K20 is generally associated with transcriptional repression, whereas methylation of H3K4, H3K36, and H3K79 is associated with transcriptional activation [39]. Reduced H3 Lys-4 methylation and increased H3 Lys-9 methylation play a critical role in the maintenance of promoter DNA methylation-associated gene silencing in colorectal cancer [40]. In HP1-mediated heterochromatin formation, the Suv39 methyltransferases promote mono-, di-, and trimethylation of H3K9. As a result, HP1 is recruited to chromatin and causes silencing of pericentromeric heterochromatin [38].

Other histone modifications

There are some other histone modifications such as histone phosphorylation, ubiquitination, sumoylation, histone poly-ADP ribosylation, histone biotination, citrullination, and proline isomerization. There is also crosstalk among the various histone modifications that can be positive or negative. Positive crosstalk refers to the interaction between various modifications for another modification to occur. For example, in *Saccharomyces cerevisiae*, Snf1 and Gcn5—the enzymes that phosphorylate H3-S10 and acetylate H3-K14, respectively—appear to work synergistically to mediate gene activation [41]. Negative crosstalk refers to competition for the same amino acid for modification. An example is methylation of H4R3 by PRMTI which impairs acetylation of H4 on K5, K8, K12, and K16 [42].

Non-Coding RNA

The RNA that does not code for a protein is called ncRNA. Unlike in eukaryotes, ncRNA forms a small part of the prokaryotic genome. Small ncRNA have been reported in bacteria. The majority of

the eukaryotic genome, however, is transcribed into ncRNA. ncRNA plays a major role in regulation of mRNA translation. It has also been reported that the small non-coding RNA 7SK regulates transcription of cyclin T/CDK9 [43]. It binds to and inhibits the activity of cyclin T complexes. ncRNA and its associated enzymes also play a role in chromosome segregation. Dicer, which cleaves dsRNA into small interfering RNA (siRNA), has a vital role to play in yeast chromosome segregation [44]. ncRNA is also important for neurogenesis and neuropsychiatric disorders [45].

ncRNA can be divided into three groups: small, medium, and long ncRNAs. Small ncRNAs are less than 200 nucleotides in length. miRNAs, siRNAs, or PIWI-interacting RNAs (piRNAs) are some of the important small non-coding RNAs that play important roles in several biological processes, especially in cancer. Medium ncRNAs are 60–300 nucleotides in length and are exemplified by small nucleolar RNA (snoRNA). Long non-coding RNAs (lncRNAs), on the other hand, are RNAs more than 200 nucleotides long. They are also called macroRNAs or long intergenic ncRNAs (lincRNAs). Recently, ncRNAs have been increasingly used for therapies. ncRNAs such as miRNAs and lncRNAs are important in the induction, maintenance, and directed differentiation of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) [46]. miRNAs also hold immense promise in the field of cancer therapy and are being explored to treat a variety of diseases.

microRNA

Small RNA molecules, miRNAs are approximately 22 nucleotides long and can negatively control their target gene expression posttranslationally. There are currently more than 2000 human miRNAs known. The expression of miRNA has been linked to cancer development. miRNAs are transcribed by RNA polymerase II into primary miRNAs and are then processed in the nucleus by RNase III Drosha and DGCR8 into precursor miRNAs. They are then exported into the cytoplasm by exportin 5 where they are cleaved by Dicer into final functional mature miRNAs. miRNAs bind to their target with either complete complementarity or partial complementarity. Complete complementarity can lead to target degradation, whereas incomplete complementarity, often in the 3′ UTR regions, leads to translational repression of their target genes. Each mRNA may be regulated by more than one miRNA.

Recently, miRNAs have been linked to cancer and can act as either oncomirs or tumor suppressor miRNAs. For example, overexpression of *miR-21* has been observed in breast cancer [47] and glioblastoma. *miR-372* and *miR-373* are upregulated in testicular germ cell tumors [48]. In *Eμ-miR-155* transgenic mice, *miR-155* has been shown to target HDAC4, which causes disruption of the BCL6 transcriptional machinery and leads to upregulation of survival and proliferation genes in *miR-155*-induced leukemias [49]. miRNA expression profiles can be used to classify human cancers. siRNAs, often considered to be closely related to miRNAs, have been shown to be involved in both DNA methylation and histone modification. The processing pathways of siRNA and miRNA share many of the enzymes involved in the RNAi pathway. Studies suggest that they affect histone modification [50]. miRNAs also act as tumor suppressors; *miR-124a* potentially targets CDK4, CDK6, cyclin D2, and EZH2 and suppresses growth, migration, and invasion of cells in uveal melanoma cells. Recent studies reveal that the miRNA gene *hsas-mir-9-1* is inactivated due to aberrant hypermethylation in breast cancer cell lines [51].

miRNAs bind to their target mRNAs and downregulate their stability and translation. Recent studies revealed that *miR-322* downregulates the expression of *Tob2* and modulates *osterix* mRNA stability. Epigenetic silencing of *miRNA-203* enhances ABL1 and BCR-AL1 oncogene expression. Aberrant hypermethylation events in the regulatory regions of miRNA play a role in human metastasis. MeCP2, a DNA

methyl-CpG-binding protein, can epigenetically regulate specific miRNAs in adult neural stem cells [52]. A very recent study revealed that *miR-17* downregulates TIMP3 in prostate cancer cell lines [53].

lncRNAs also play a variety of roles in disease. For example, the lncRNA *MALAT1* is upregulated in cancer tissues, and in human cells it promotes cellular proliferation by modulating the expression and/or pre-mRNA processing of cell-cycle-regulated transcription factors [54]. *Hox* genes are associated with hundreds of lncRNAs that define domains of differential histone methylation and RNA polymerase accessibility along the spatial and temporal axes of human development [55]. A lncRNA transcribed from the *HOXC* locus, termed *HOTAIR*, is upregulated in non-small cell lung cancer. HOTAIR downregulates HOXA5, resulting in increased cell invasion and lymph node metastasis [56]. lncRNAs are believed to bind to chromatin and then recruit chromatin-modifying complexes [57]. lncRNAs operate to activate gene expression through trithorax-group complexes [58]. lncRNAs are also frequently associated with the phenomenon of genomic imprinting, which ensures that one of the two parental alleles of certain autosomal genes is epigenetically silenced [59].

Correlation of epigenetics with genetics, transcriptomics, and proteomics

Epigenetics is a complex process, and its study must complement the study of genetics, proteomics, and transcriptomics. The transcriptome involves the vast array of RNAs such as miRNA, lncRNA, and short hairpin RNA that also have epigenetic roles. Among the important proteins involved in epigenetic processes are the histone proteins. There is a constant interaction between the epigenetic machinery and proteins such as transcription factors that attempt to bind with the methylated DNA, chromatin remodeling factors, various enzymes such as histone acetylases, and methyltransferases. Hence, a holistic study of epigenetics will encompass the study of proteomics and transcriptomics.

In addition to acetylation and methylation, histones undergo other posttranslational modifications such as ubiquitination, phosphorylation, sumoylation, and poly(ADP-ribosyl)ation. There are more than 40 variants of human histones known. Quantitative mass spectrometry (MS) is a versatile technique to elucidate the chemical structures of molecules such as peptides. It is used extensively in epigenetics to study histones, chromatin remodeling proteins, and other posttranslational modifications of histones. There are three common MS methods for characterizing histones. In the first one, known as the bottom-up approach, proteins are cleaved into smaller amino acids and then analyzed by MS [60]. The second approach, referred to as top down, does not involve cleaving the protein and analyzes intact proteins [61]. The third strategy, known as middle down, involves cleaving the N-terminal tail of histones and then analyzing it using MS [62]. The endogenous level of acetylation was observed at individual lysine residues in histone H4 by using stable isotope labeling [63]. Others have proposed a method that combines fast sample preparation with relative quantitation by MS by labeling histones with propionic anhydride [64]. Recently, positron emission chromatography has begun to be used to image HDACs and their antagonists [65]. Another method to study proteins and their interaction with DNA is known as the chromatin immunoprecipitation (ChIP) assay. The more advanced form of ChIP, called ChIP-seq, combines chromatin immunoprecipitation with high-throughput, next-generation DNA sequencing technologies and can render genome-wide profiles of epigenetic modifications such as positions of histones with specific modification of their N-terminal tails [66]. Recent studies using ChIP-seq revealed that *Arabidopsis* telomeres exhibit euchromatic features and are labeled with H3K27me3 [67]. Combining

ChIP-seq with massively parallel sequencing showed that nuclear Ago1 directly interacts with RNA polymerase II and is widely associated with chromosomal loci throughout the genome with preferential enrichment in promoters of transcriptionally active genes [68]. The advances in next-generation sequencing combined with chromatin immunoprecipitation will help in the elucidation of many important proteins involved in epigenetic processes.

A further layer of complexity is added to the epigenetic process at the transcriptional level. Subtle changes in splice site variations and 3′ untranslated regulatory regions significantly affect gene expression patterns. ncRNAs are also involved in various epigenetic modifications; therefore, investigation of the transcriptome can reveal the expression levels of various genes involved in the epigenetic processes. An emerging field of transcriptomics is expression profiling of genes encoding miRNAs. Antagomirs targeted to *miR-21* reduced remodeling, and *miR-23a* knockdown in mice prevented cardiac hypertrophy [69]. In a recent study, advanced RNA sequencing was used to detect the hepatic mRNA expressions of various epigenetic modifiers at different ages of c57BL/6 mice [70]. Thus, the study of epigenetics is incomplete without a proper study of proteomics and transcriptomics. Exploring the interrelationships among genetics, epigenetics, transcriptomics, and proteomics will help us understand these complex mechanisms more fully and open new avenues for therapy and diagnoses.

Clinical and medical implication of epigenetics

Aberrations in epigenetic processes may be a cause of a number of diseases. For example, alteration in DNA methylation can cause imprinting diseases such as Prader–Willi and Angelman syndromes. Cancer is the mostly widely studied disease caused by epigenetic mechanisms. Studies show that DNA methylation and histone modifications contribute to many other diseases such as diabetes, obesity, allergic disorder, cardiovascular disease, and neurological disease. Environmental toxins also modify epigenetic patterns and cause changes in behavior and development. A very recent study on BALB/C mice [71] demonstrated behavioral changes in mice upon administration of bisphenol A (an endocrine disruptor) in the gestation period. This toxin not only changed the maternal behavior of mice but also caused significant changes in the offspring. The mice also showed increase levels of DNMT1 and DNMT3a expressions in their *esr1* and *esr2* genes, implying that methylation changes may be the cause of their strange behavior. Aging is also known to be caused by epigenetic processes.

DNA methylation causes silencing of many genes such as tumor suppressor genes. CpG islands, which are generally found in the promoter region of the genes, are CG-rich regions. When they are methylated, they may cause the transcriptional silencing of the genes. Histone modifications also play a role in disease. Modifications such as acetylation, which signifies transcriptional activity, may cause silent genes such as oncogenes to become active. Table 2.1 summarizes a list of diseases and the epigenetic mechanisms causing the diseases.

Epigenetic diseases have paved the way for novel modes of treatment called epigenetic therapy. Epigenetic modifications are generally reversible, and epigenetic therapy involves processes that can alter the epigenetic aberrations. Inhibitors of DNA methylation such as 5-azacytidine (5-aza-CR) and 5-aza-2′-deoxycytidines (5-aza-CdR) can cause these genes to be re-expressed and can be used as treatment [72,73]. These drugs are nucleoside analogs that incorporate into DNA in place of cytosine. Moreover, the U.S. Food and Drug Administration (FDA) has approved the use of these two drugs for the selective treatment of a preleukemic disease, myelodysplastic syndrome [74]. Other drugs such as

Table 2.1 Diseases and Their Associated Epigenetic Mechanisms

Disease	Epigenetic Mechanism	Ref.
Cancer	Silencing of tumor suppressor genes or activation of oncogenes through DNA methylation and histone modifications mediated by miRNA	Sharma et al. [79]
Rheumatoid arthritis	Global hypomethylation	Karouzakis et al. [80]
Systemic lupus erythematosus	T cells having global DNA hypomethylation	Richardson et al. [81]
Type II diabetes	Increased DNA methylation and decreased expression of PDX-1 in pancreatic islets	Yang et al. [82]
Congenital heart disease	Dysregulation of DNA methylation during different stages of embryonic growth	Chowdhury et al. [83]
Obesity	Loss of function of the histone demethylase Jhdm2a	Inagaki et al. [84]
Depression	Reduced expression of glucocorticoid receptor (GR) through increased DNA methylation in the promoter of GR	Krishnan and Nestler [85]
Asthma	DNA methylation of genes critical to T-helper (Th) differentiation	Miller and Ho [86]
Huntington's disease	H3K9 hypertrimethylation	Akbarian and Huang [87]
Alzheimer's disease	Histone acetylation and phosphorylation	Francis et al. [88]
Beckwith–Wiedemann syndrome	Aberrant methylation and imprinting of H19, a gene for long non-coding RNA	DeBaun et al. [89]
Rubinstein–Taybi syndrome	Altered CBP/p300 function	Abel and Zukin [90]

depsipeptide and valproate inhibit histone deacetylases and can be used for the treatment of leukemia [75]. miRNAs also play a role in a variety of diseases. They function both as oncogenes and as tumor suppressor genes. *MiR-21* is an oncogene, and knockdown of such miRNAs can cause apoptosis and prevent cancer. Modified antisense oligonucleotides (ASOs), which are short synthetic analogs of natural nucleic acids complementary to miRNA, can be used to inhibit oncogenic miRNA and treat a variety of diseases. Modified oligonucleotides have been used to target human oncogene *Ha-ras* and inhibit T-24 cell proliferation [76]. Krützfeldt et al. [77] intravenously administered a cholesterol-conjugated 2′-*O*-methyl-modified ASO referred to as antagomir into mice and reported *in vivo* reduction of mature *miR-122* with no effect on *pre-miR-122* levels. A new strategy has been employed for treating hepatitis C virus by using oligonucleotide inhibitors of *miR-122* [78]. These and many other new therapies require the understanding of the molecular mechanisms of epigenetics. Collectively, it is apparent that the epigenome holds tremendous potential for therapeutics and can revolutionize approaches in medical biology.

Conclusion

Our understanding of epigenetic mechanisms and the roles they play in disease processes is still in its infancy. Epigenetic change is caused by a variety of factors, among which diet and environment play an important role. In order to eliminate the deleterious effects of epigenetics we need to understand

the factors that cause these changes. Manipulating the epigenome holds enormous promise for preventing and treating human illness. Recent advances in technologies to study epigenetic mechanisms have broadened our understanding of development of disease. Deeper insight into epigenetic processes can provide valuable information on possible targets for pharmacological research.

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Glossary

Chromatin immunoprecipitation assay A method used to identify the DNA sequence to which proteins such as histones are bound. It uses specific antibodies to immunoprecipitate the proteins.

Chromatin remodeling The change in the accessibility of DNA by transcription factors brought about by changes in the position of histones and DNA.

CpG island Cytosine–phosphate–guanine dinucleotide-rich region of the genome usually located in and around the promoter region of genes.

***De novo* methylation** A process mediated by DNMT 3 methyltransferases that involves the establishment of methylation patterns during embryonic development.

Demethylases The enzymes that catalyze the removal of a methyl group from 5-methylcytosine and also regulate the chromatin state.

DNA methylation An epigenetic modification that involves the covalent addition of methyl groups to the carbon 5 of the cytosine ring, generally in CpG dinucleotides.

DNA methyltransferase The enzymes that aid the process of addition of a methyl group to position 5 of the cytosine pyrimidine ring. DNMT 1 and DNMT 3 are the major human DNA methyltransferases.

Epigenetic change Heritable changes in the DNA that do not alter the nucleotide sequence but can regulate the expression of genes.

Euchromatin The loosened state of chromatin that is favorable for transcription. It is generally associated with histone acetylation and non-methylated DNA.

Heterochromatin The condensed state of chromatin not accessible to transcription factors. It is generally associated with methylated DNA regions bound by histone deacetylases.

Histone acetyltransferases The enzymes that catalyze the addition of acetyl groups to specific lysine residues in the histone tails.

Histone deacetylase The enzymes that remove the acetyl group from specific lysine residues of the histone tail.

Histone modification Posttranslational modification of histones that regulates the chromatin structure. The modifications include acetylation, methylation, and phosphorylation, to name a few.

Long ncRNAs (lncRNA) The RNA transcripts that are more than 200 nucleotides long and believed to bind to chromatin and recruit other chromatin-modifying complexes.

Methyl-CpG-binding protein 2 (MeCP2) Protein with methyl-binding domains that binds to methylated CpG islands and may recruit chromatin remodeling complexes and influence acetylation of nearby histones.

MicroRNA (miRNA) Non-coding RNAs that are 22–23 nucleotides long; they bind to the 3′ untranslated region of specific mRNAs and regulate the expression of certain genes.

Non-coding RNA RNA molecules that are not translated into proteins. They can epigenetically modify a variety of cellular processes. Some non-coding RNAs important in epigenetics are microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and large non-coding RNAs (lncRNAs).

SiRNA Double-stranded RNA molecules that are 21 nucleotides long and bind to specific mRNA molecules, often resulting in their degradation.

TET proteins Ten-eleven translocation 1–3 (TET1–3) proteins catalyze the conversion of 5-methylcytosine into 5-hydroxymethylcytosine.

X inactivation The epigenetically mediated inactivation of one of the X chromosomes in females, often due to formation of a heterochromatin state.

CHAPTER

A Brief History of Epigenetics in Psychiatry

3

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KEY CONCEPT

- This chapter discusses the history of epigenetics as it pertains to psychiatry.

ABBREVIATIONS

CpG Cytosine–phosphate–guanine
FMR1 Fragile X mental retardation 1 gene
MeCP2 Methyl-CpG-binding protein 2

Introduction

In the first half of the twentieth century, developmental biology and genetics were separate disciplines [1]. Toward the middle of the twentieth century, a few leading biologists, such as Conrad Waddington and Ernst Hadorn, realized that genetics and developmental biology were related to each other and should eventually come together in a common discipline [1]. Waddington, the founder of the field of

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epigenetics, removed the distinction between embryology and genetics and coined the term epigenetics in the 1940s [2]. The term was a neologism that combined the words "epigenesis" (embryonic development) and "genetics" [2]. Waddington defined epigenetics as "the branch of biology which studies the causal interaction between genes and their products which brings the phenotype into being" [3].

Epigenetics is attracting ever-increasing interest in medicine, including psychiatry, among both researchers and clinicians. At present, a large amount of experimental work is being carried out on epigenetics in psychiatry throughout the world. Findings from research on epigenetics in psychiatry are often reported in the popular media. It has been suggested that in the future epigenetics may be of crucial importance in psychiatry [4]. This chapter discusses in brief how epigenetics in psychiatry has evolved over the decades.

Erikson's epigenetic principle

Epigenetics was discussed in relation to psychology and psychiatry as early as the middle of the twentieth century by the noted psychoanalyst and developmental psychologist Erik Erikson (1902–1994). Erikson was born in Germany but emigrated to the United States in 1933. Over his long life he worked initially as a teacher, next as a child psychologist, then as an anthropological field worker, and finally as a biographer [5]. Erikson is one of the most eminent and highly cited and discussed psychologists in the history of psychology [6].

In 1950, Erikson published *Childhood and Society*. In this publication, he suggested a psychosocial theory of development that describes important steps in an individual's relationships with the social world. This theory was original and became a highly influential theory of psychological development and crisis occurring in periods that extended across the entire life cycle [5]. In this publication, Erikson presented clinical cases in which individual psychodynamics, society, and history are interlinked with a skill not seen before or after [7]. The work includes analyses of children's play and development in different cultures, a theoretical sketch of the entire human life cycle, pieces on the problems of social identity, and biographical essays of Adolf Hitler and the Russian writer Maxim Gorky [7].

Erikson's concept of psychosocial development across life was the centerpiece of his work, and he expanded on this concept throughout his later writings. It takes as its model epigenesis, a term obtained from embryology referring to the unfolding development in an organism, especially the development of a plant or an animal from an egg or spore by a series of steps, during which cells differentiate and organs form. According to Erikson, there are eight stages of the life cycle, with individual aspects of development proceeding according to a predetermined timetable (Table 3.1). Elements of each stage are present from birth and differentiate over time. Each of the stages has its own period of quiescence and critical ascendency. Each stage depends on the proper development of the other stages in the correct sequence. Each of the stages presents the individual with tasks to be achieved. Failure to resolve the conflicts of a particular stage makes coping at later stages more difficult. Work on any particular stage is never complete, and old developmental conflicts can be activated by important life events [8].

Erikson did not discuss the possible molecular mechanisms underlying the epigenetic principle he propounded. In 2012, Peedicayil compared Erikson's epigenetic principle with epigenetics and suggested that the two entities have many aspects in common with each other [8]. Peedicayil further suggested that if Erikson's epigenetic principle is valid and credible then the underlying molecular mechanisms would be those underlying epigenetics [8].

Table 3.1 Summary of Erikson's Epigenetic Principle Involving Eight Psychosocial Stages of the Life Cycle

Stage No.	Psychosocial Stage	Life Stage	Approximate Age (Years)
1	Trust vs. mistrust	Infancy	0–1½
2	Autonomy vs. shame and doubt	Early childhood	1½–3
3	Initiative vs. guilt	Play age	3–6
4	Industry vs. inferiority	School age	6–13
5	Identity vs. role confusion	Adolescence	13–20
6	Intimacy vs. isolation	Young adulthood	20–40
7	Generativity vs. stagnation	Adulthood	40–60
8	Integrity vs. despair	Late adulthood	60+

Source: Peedicayil J. A comparison of Erikson's epigenetic principle with epigenetics. J Theor Biol 2012; 315: 144–5. With permission.

Schizophrenia: the epigenetic puzzle

In 1982, the American psychiatric geneticist Irving Gottesman, along with his colleagues James Shields and Daniel Hanson, published an important and highly cited book, *Schizophrenia: The Epigenetic Puzzle* [9]. In this book, the authors suggested that schizophrenia, being a common genetic disorder, is likely to be an epigenetic puzzle involving the interaction of genes and the environment. They suggested that both genes and the environment are essential for the development of the disorder and the task ahead was to discover how genes and the environment interact to produce the disorder. The authors discussed various genetic aspects of the epigenetic puzzle: family studies, twin studies, and adoption studies in patients with schizophrenia. They then discussed various environmental aspects of the epigenetic puzzle: life events, obstetric complications, and combinatorial environmental factors in patients with schizophrenia. The authors also discussed the possible role of autism and childhood schizophrenia in the epigenetic puzzle of schizophrenia. Finally, they discussed various aspects of the social biology of patients with schizophrenia: sexuality, marriage, fertility and divorce, and patterns of mortality and morbidity. However, despite the popularity of this book, it did not stimulate the study of molecular epigenetic studies on schizophrenia [10].

Later theoretical developments

Around the turn of the century, other authors also discussed the possibility of an epigenetic basis for the non-organic psychiatric disorders. Of these authors, Petronis and colleagues [10,11] suggested that, in addition to DNA sequence changes, epimutations could contribute to the pathogenesis of schizophrenia, and that epigenetic factors underlying schizophrenia should be investigated. A little later, Petronis [12] suggested that epigenetic factors could be important to the development of the major psychoses (schizophrenia and bipolar disorder). The following year, the same author suggested [13] that an epigenetic basis for the major psychoses is more consistent with many of the clinical features of these disorders than is a genetic basis based on DNA sequence variation alone. Peedicayil [14,15] provided

evidence from studies on human evolution that suggested that the non-organic psychiatric disorders are epigenetic, rather than genetic, disorders. He suggested that epimutations, rather than genetic mutations, underlie these disorders. Crow [16] suggested that the basis for major psychosis may be epigenetic in nature and related to the development of language in humans. He further suggested that the basis for major psychosis may lie in the interaction between genetic and epigenetic mechanisms that distinguished the course of the evolution of the brain in *Homo sapiens* from that in other primates. Later, Peedicayil [17] provided evidence based on studies on rodents to suggest that psychosocial factors may act by altering epigenetic mechanisms of gene expression in the pathogenesis of psychiatric disorders.

Experimental (laboratory) studies on epigenetics in psychiatry

Experimental (laboratory) studies on the epigenetics of organic psychiatric disorders began before those on the non-organic psychiatric disorders. The former have also been more successful than the latter. The molecular basis of fragile X syndrome was elucidated by positional cloning in 1991 and found to be associated with a large trinucleotide repeat expansion in the fragile X mental retardation 1 (*FMR1*) gene [18–20]. This discovery revealed one of the first human disorders caused by dynamic mutations. The *FMR1* gene is comprised of 17 exons and is about 38 kilobases long. In the 4.4 kilobases of the *FMR1* transcript a CGG trinucleotide repeat is found in the 5′ untranslated region (5′UTR) [21]. In normal individuals, this repeat is very polymorphic in length and often punctuated by AGG interruptions. Normally, the repeat size ranges from 7 to about 60, with 30 repeats found on the most common allele. In most affected patients, the repeats are highly expanded over 230 repeats and become abnormally hypermethylated, leading to silencing of the *FMR1* gene [21].

Epigenetic abnormalities have also been elucidated in other organic psychiatric disorders. For example, Huda Zoghbi and colleagues discovered in 1999 that Rett syndrome is caused by mutations in the gene *MeCP2* encoding X-linked methyl-CpG-binding protein 2 (MeCP2) [22]. More details of the data acquired on epigenetic abnormalities in the organic psychiatric disorders are discussed in Chapters 17 to 20. Experimental studies on epigenetic abnormalities in patients with non-organic psychiatric disorders such as schizophrenia and bipolar disorder have been conducted since around the middle of the first decade of this century, and this area of research is increasingly attracting interest. Although the majority of experimental studies on epigenetic abnormalities in patients with non-organic psychiatric disorders have focused on abnormalities of DNA methylation, such studies have also involved investigation of abnormalities in modifications of histones and abnormalities of RNA-mediated regulation of gene expression. Such studies have investigated postmortem brains as well as peripheral tissues obtained from patients with non-organic psychiatric disorders. There are inherent difficulties associated with these studies [23,24]. It is not clear whether some of the epigenetic changes that are detected in patients with the non-organic psychiatric disorders are the cause or the effect of underlying disease process. There is a lot of variability in epigenetic patterns within the same individual, between individuals, and between cohorts; experimental conditions used by researchers can influence the results of studies; the use of nicotine and psychopharmacological drugs can influence epigenetic patterns of subjects; and there is variability in epigenetic patterns between different neurons within the brain. More details of the results of laboratory studies on patients with non-organic psychiatric disorders are discussed in Chapters 10 to 16.

The social environment and epigenetics in psychiatry

As early as 1999, the behavioral scientist Michael Meaney and his colleagues showed that variation in maternal care in rats could influence the development of behavioral and endocrine responses to stress in the offspring. Based on cross-fostering studies in rats, their work provided evidence for a causal relationship between maternal behavior and reaction to stress in the offspring and the transmission of individual differences in maternal behavior in female rats from one generation to the next. Moreover, they showed that an environmental manipulation imposed during early development that changed maternal behavior could alter the pattern of transmission in later generations. Hence, their data suggested non-genomic transmission of maternal behavior and stress response in rats [25]. The authors did not discuss the precise molecular mechanisms involved. Subsequently, Meaney teamed up with the epigeneticist Moshe Szyf, and together they published an important study in 2004 which showed that maternal behavior in rats, such as licking, grooming, and arched-back nursing, altered the epigenome of pups at a glucocorticoid receptor promoter in the hypothalamus and that these changes could be reversed [26].

Later, Meaney and Szyf joined forces with the psychiatrist Gustavo Turecki and extended the findings on rats to humans. They examined epigenetic differences in the same promoter of the same gene as in the study on rats in the hippocampus between suicide victims with a history of childhood abuse and those without such a history or controls. They found epigenetic differences in the promoter of this gene between suicide victims with a history of childhood abuse and the other two study groups, providing evidence that epigenetic regulation of the glucocorticoid receptor gene is associated with childhood abuse [27]. More details about these studies are discussed in Chapter 26.

Conclusions

This chapter has provided a brief outline of the history of epigenetics in psychiatry. After progressing gradually for several decades, the pace of research regarding epigenetics in psychiatry has markedly increased over the past decade. However, it remains to be seen whether, and to what extent, epigenetics in psychiatry impacts the practice of clinical psychiatry and improves the clinical management of patients with psychiatric disorders. The goal of research on epigenetics in psychiatry should be to improve the clinical management (prevention and treatment) of psychiatric disorders.

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Glossary

Major psychosis A type of psychiatric disorder that mainly includes schizophrenia and bipolar disorder.
Non-organic psychiatric disorder A psychiatric disorder not due to any known underlying brain dysfunction.
Organic psychiatric disorder A psychiatric disorder due to transient or permanent brain dysfunction due to specific organic factors such as aging, trauma, or tumors.

CHAPTER

Roles of Epigenetics in the Neural Stem Cell and Neuron

4

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KEY CONCEPTS

- During cortical development, neural stem cells sequentially acquire abilities to differentiate into neurons and glial cells in response to combinations of spatiotemporally regulated extrinsic factors and cell-intrinsic epigenetic changes.
- Combinations of *trans*-acting epigenetic modifiers can modulate the global and local opening and closing of the chromatin structure, which is also affected by the initial epigenomic status.
- Postmitotic neurons show significant plasticity of their epigenomic profiles in response to transient extrinsic stimuli.
- The epigenomic profiles of neurons define a much more robust identity than those of other neural cell types.

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ABBREVIATIONS

3C Chromosome conformation capture
5caC 5′-Carboxylcytosine
5hmC 5′-Hydroxymethylcytosine
5hmU 5′-Hydroxymethyluracil
5mC 5′-Methylcytosine
AID Activation-induced deaminase
APOBEC Apolipoprotein B mRNA-editing enzyme complex
Ascl1 Achaete–scute complex homolog 1
BDNF Brain-derived neurotrophic factor
BER Base excision repair
bHLH Basic helix–loop–helix
BMP Bone morphogenetic protein
Brg1 Brahma-related gene 1
CaMK Ca^{2+}/calmodulin-dependent kinase
CaN Calcineurin
CBP CREB-binding protein
ChIP-seq Chromatin immunoprecipitation sequencing
cKO Conditional knockout
CNTF Ciliary neurotrophic factor
CpG Cytosine–phosphate–guanine
CREB cAMP response element-binding protein
Ctip2 COUP-TF interacting protein 2
DAXX Death-domain associated protein
DG Dentate gyrus
DIV Days *in vitro*
DL Deep layer
Dlx2 Distal-less homeobox 2 gene
Dm-20 Neuronal membrane glycoprotein M6-b-like
DNMT DNA methyltransferases
dsRNA Double-stranded RNA
E11 Embryonic day 11
Eed Embryonic ectoderm development
ERK Extracellular signal-regulated kinase
ESCs Embryonic stem cells
Eset ERG-associated protein with SET domain
Ezh2 Enhancer of zeste homolog 2
FGF Basic fibroblast growth factor
FKBP5 FK506 binding protein 5
GAD67 Glutamate decarboxylase 67
GFAP Glial fibrillary acidic protein
GLP G9a-like protein
Gp130 Glycoprotein 130
GRM Metabotropic glutamate receptor
H3K27me3 Trimethylation at lysine 27 on histone H3
H3K9 Histone H3 lysine 9
HAT Histone acetyltransferase
HDAC Histone deacetylase
HES Hairy and enhancer of split
Id Inhibitor of differentiation
IL-6 Interleukin 6
iN cell Induced neuronal cell
iPSC Induced pluripotent stem cell
JAK Janus kinase
JMJD Jumonji C domain-containing histone demethylase
LIF Leukemia inhibitory factor
lncRNA Long non-coding RNA
LSD1 Lysine-specific H3K4 demethylase 1
LTP Long-term potentiation
MBD Methyl-CpG-binding domain
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
Mll1 Mixed-lineage leukemia 1
N-CoR Nuclear receptor corepressor
ncRNA Non-coding RNA
NCSs Neural stem cells
Ngn Neurogenin
NMDA *N*-methyl-D-aspartate
NPCs Neuronal precursor cells
NR3C1 Neuron-specific glucocorticoid receptor
NRSE/RE1 Neuron-restrictive silencer element/RE-1
NRSF/REST Neuron-restrictive silencing factor/RE-1 silencing transcription factor
NSC Neural stem cell
OPC Oligodendrocyte precursor
OSKM *Oct3/4*, *Sox2*, *Klf4*, and c-*Myc*
pancRNAs Promoter-associated non-coding RNA
P10 Postnatal day 10
PcG Polycomb group
PLP Myelin proteolipid protein
PP1 Protein phosphatase 1
PRC Polycomb repressive complex
RA Retinoic acid
Ring1B Ring finger protein 1B
RMST Rhabdomyosarcoma 2-associated transcript
S100β S-100 calcium-binding protein, beta chain
SAHA Suberoylanilide hydroxamic acid
Satb2 Special AT-rich sequence-binding protein
Shh Sonic hedgehog
Sirt Sirtuin
SMAD1 Mothers against decapentaplegic homolog 1
Sox SRY-box containing gene
STAT Signal transducer and activator of transcription
SVZs Subventricular zones
SWI/SNF SWItch/Sucrose NonFermentable
T-DMR Tissue-dependent and differentially methylated region
TET Ten-eleven translocation
TSA Trichostatin A
UL Upper layer
VPA Valproic acid
VZ Ventricular zone
Wnt Wingless-type
YY1 Yin Yang 1

Outline of neural cell production during cortical development

Differentiated cells in the mammalian brain are generated from neural stem cells (NSCs) that divide repeatedly in the ventricular zone (VZ) of the embryonic brain. After birth, NSCs are located in restricted areas such as the early postnatal and adult subventricular zones (SVZs) of the forebrain and hippocampal dentate gyrus (DG). NSCs exhibit two defining characteristics: the capacities for self-renewal and for generating specialized cell types (i.e., neurons, astrocytes, and oligodendrocytes). These capacities are controlled spatiotemporally. For example, from embryonic day 11 (E11) to E18, NSCs preferentially produce neurons in the mouse cerebral cortex [1]. During the generation of these cortical neurons, Cajal–Retzius neurons forming the preplate are produced first. The preplate is then separated into the marginal layer, which later becomes cortical layer I, and the subplate layer. Then, between these two layers, cortical layer II–VI neurons are generated in an inside-out order as development proceeds: The deep layer VI and V neurons are produced first, followed by the more superficial layer IV, III, and II neurons. In parallel, NSCs gradually acquire the capacity to generate astrocytes [1]. The majority of mouse oligodendrocytes are generated after birth in the cerebral cortex (Figure 4.1).

These sequential steps enable the initial establishment of neuronal networks followed by integration of glial cells that support the functioning of the neuronal networks. In the following four sections, we summarize the spatiotemporal fate determination mechanisms of NSCs controlled by epigenetic regulation, focusing especially on mouse cortical development.

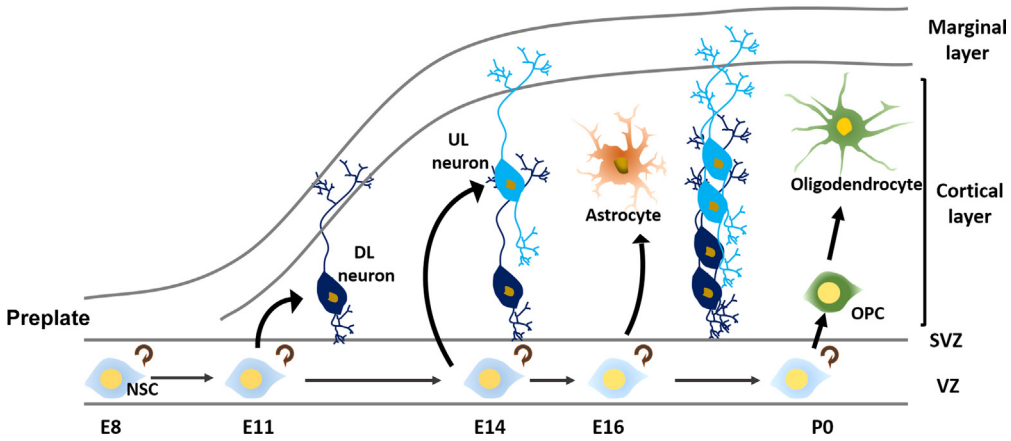

FIGURE 4.1

Schematic representation of neural cell production during mouse cortical development. Starting from E8 until E11, prior to neurogenesis, NSCs undergo self-renewal and expand their pool. Following the onset of neurogenesis, around E11, the NSCs become competent for generating neurons, and start producing deep layer (DL) neurons. Around E14, NSCs switch to producing upper layer (UL) neurons and also acquire potency to differentiate into astrocytes prior to their production. At late embryonic stage, around E16, NSCs start generating astrocytes. After birth, NSCs mainly produce glial lineage cells. At this stage, some OPCs, which had already existed from an early cortical developmental stage, start to generate oligodendrocytes.

Epigenetic regulation of exit from cell proliferation cycle toward postmitotic neurons during early cortical development

In E8 mouse cortical NSCs, ectopic expression of stabilized β-catenin (a constitutively active mutant that activates the canonical wingless-type [Wnt] pathway) promotes proliferation of NSCs, resulting in an enlarged embryonic cortex. In contrast, in E11–E13 mice, activation of the canonical Wnt pathway signal induces NSC differentiation into neurons through the generation of transiently amplifying neuronal precursor cells (NPCs) by upregulation of the proneural basic helix–loop–helix (bHLH) transcription factor neurogenin1 gene (*Ngn1*) [2]. Variation in the patterns of methylation of histone tails, which reflect and modulate chromatin structure and function, contribute to this stage-dependent regulation of different responses to extracellular Wnt signals. For example, trimethylation at lysine 27 on histone H3 (H3K27me3) is associated with transcriptional repression of neighboring genes in early neurogenesis via changing the expression of enhancer of zeste homolog 2 gene (*Ezh2*), a histone H3K27 methyltransferase that participates in polycomb group (PcG) protein-containing polycomb repressive complex 2 (PRC2) together with embryonic ectoderm development (Eed). In E8–E10 NSCs, *Ezh2* is highly expressed, and prevents Wnt-signal-mediated activation of neuronal genes and thus blocks neuronal differentiation. After E11, decreasing levels of Ezh2 expression gradually cause increased Wnt-signal-mediated neuronal differentiation of NSCs. The contribution of the decrease in *Ezh2* expression to acquisition of the neuronal differentiation capacity of NSCs is supported by the fact that NSC-specific *Ezh2* conditional knockout (cKO) mice overproduce early-born neurons from NSCs at the expense of self-renewal at early embryonic stages [3,4]. Interestingly, a long non-coding RNA (lncRNA) named *utNgn1* is transcribed from 5.8 to 7.0 kb upstream of mouse *Ngn1* in the E11 cortex, and its expression seems to be regulated by Ezh2. The expression of *utNgn1* is highly correlated with that of *Ngn1* during neuronal differentiation of NSCs. Knockdown of *utNgn1* caused repression of *Ngn1*. Furthermore, the amounts of *utNgn1* and *Ngn1* were increased by Wnt signal activation and were downregulated by binding of PRC2 to H3K27me3 in the *utNgn1* region [5]. These results suggest that decreasing Ezh2 converts the epigenetic ability of the *Ngn1* locus in NSCs to sense the Wnt signaling toward neuronal differentiation through *utNgn1* upregulation. *Ngn1* is not the sole target of Ezh2, because chromatin immunoprecipitation sequencing (ChIP-seq) analysis showed that in E14 proliferating embryonic NSCs, Ezh2 preferentially binds not only to the *utNgn1* coding genomic region but also to the promoters of a set of neural differentiation-associated genes, such as the neurogenic differentiation 2, T-cell leukemia homeobox 3, and phosphonoacetaldehyde hydrolase 2b genes [4].

Methylation at lysine 9 on histone H3 (H3K9), which is generally associated with transcriptional repression of nearby genes, is also essential for early neuronal differentiation. ERG-associated protein with SET domain (ESET, also called Setdb1), a histone H3K9 methyltransferase, is highly expressed in proliferating NS/PCs, but not in neuronal layers at early stages of mouse cortical development. *ESET* mRNA expression is gradually decreased and hardly detectable at E17, when the transition from neurogenesis to astrogenesis occurs in the dorsal cortex [6]. Forebrain-specific or NSC-specific *ESET* cKO mice showed severe brain defects and did not survive beyond postnatal day 10 (P10). In E11 *ESET* cKO mouse brain, the number of NSCs was significantly reduced. At E12, early-born layer V and VI neurons were markedly reduced in the *ESET* cKO cortex. At E13, the production rate of upper layer (UL) neurons was significantly increased, whereas that of deep layer (DL) neurons was decreased because of

cell death. Although specific gene targets of ESET remain unknown, the above-noted facts suggest that ESET is essential for both proliferation of NSCs and production of DL neurons in early neural development [6].

In contrast to H3K9 methylation, H3K4 methylation is generally associated with transcriptional activation of neighbor genes. Lysine-specific H3K4 demethylase 1 (LSD1) is highly expressed in early embryonic NSCs [7]. Inhibition of *LSD1* expression in embryonic mouse NSCs results in reduced proliferation through upregulation of cell-cycle inhibitors *p21* and phosphatase and tensin [7].

Perturbing histone acetylation status also disrupts the balance between cell proliferation and differentiation, as described below. In general, acetylation of histone tails by histone acetyltransferases (HATs) decreases the electrostatic affinity between DNA and histone, resulting in an open-chromatin structure that leads to transcriptional upregulation of genes. Conversely, histone deacetylation by histone deacetylases (HDACs) induces transcriptional suppression. HDACs can be classified into four distinct groups (classes I to IV). HDAC1, 2, 3, and 8, belonging to the class I group, are generally localized to the nucleus and expressed in ubiquitous manners in many tissues. In particular, both HDAC1 and 2 are significantly expressed in proliferating E13 NSCs [8]. Treatment of E11 NSCs with a class I and II HDAC inhibitor (valproic acid, VPA) leads to upregulation of *Ngn1* and *Ngn2* expression due to the elevation of histone acetylation levels around their promoters, resulting in promotion of neuronal differentiation [2]. In addition, it has been reported that in embryonic mouse NSCs, both HDAC3 and 5 form repressive complexes with LSD1, contributing to the repression of *p21*, which is known to inhibit cell-cycle progression [7].

Epigenetic regulation underlying properties of multipotent neural stem cells and their differentiation into upper layer cortical neurons and astrocytes

Around E14, mouse cortical NSCs start to generate primarily UL neurons [9]. Although little is known about epigenetic mechanisms of the NSC capacity switch that allows production of UL neurons instead of DL neurons, it has been reported that decreasing H3K9 methylation is involved in this switch. In fact, as described above, it has been shown that the number of UL neurons is increased, while early DL neurogenesis and self-renewal of NSCs are impaired in NSC-specific *ESET* cKO mice [6]. In addition to histone methylation, local histone acetylation status is also considered to be involved in the transition from DL neuron- to UL neuron-producing NSCs. After E13, special AT-rich sequence-binding protein (Satb2), which recruits the nucleosome remodeling and histone deacetylase complex including HDAC1, represses DL neuron marker COUP-TF interacting protein 2 gene (Ctip2) via direct binding to the matrix attachment regions in the introns [10]. *Satb2* deficiency in mice results in expansion of Ctip2-positive DL neurons, whereas the number of newly born neurons decreases in the UL between E15 and E18, which explains the significant reductions in cortical thickness at E18. In contrast, overexpression of *Satb2* in E13 NSCs markedly decreased the number of differentiated Ctip2-positive neurons [10]. Moreover, *in utero* treatment with class I and II HDAC inhibitor (suberoylanilide hydroxamic acid, SAHA) around E14 increased the production of UL neurons, and decreased that of DL neurons [11]. Considering the fact that HDAC1 represses the expression of *Ctip2*, this report implies that other class I and II HDAC family members repress genes, including genes related to UL neuron production.

Many studies have shown that cytokine signaling pathways contribute to the regulation of astrocytic differentiation from NSCs. The interleukin 6 (IL-6) family of cytokines, including ciliary neurotrophic factor, leukemia inhibitory factor (LIF), and cardiotrophin-1, activate the janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway through their interaction with a heterodimeric receptor complex of LIF receptor beta and glycoprotein 130 (Gp130), triggering the differentiation of NSCs into astrocytes [12]. Bone morphogenetic proteins (BMPs) also activate the expression of astrocytic genes via formation of a complex between BMP-downstream transcription factor mothers against decapentaplegic homolog 1 (SMAD1) and STAT bridged by a transcriptional coactivator, histone acetyltransferase p300/CREB binding protein (p300/Cbp) [12]. The NOTCH/DELTA downstream effectors hairy and enhancer of split 1 (HES1) and HES5 also activate the JAK/STAT pathway by facilitating assembly of a complex between JAK2 and STAT3 [13]. Gene knockout of either *Gp130* or *STAT3* impairs astrocyte differentiation, further indicating that JAK/STAT signaling has an essential role in astrocytic differentiation from NSCs [12].

However, despite the fact that JAK/STAT pathway-related proteins are present and are activated in the early NSCs, these cells cannot differentiate into astrocytes [12]. This can be explained by the fact that epigenetic regulation is also involved in this developmental stage-dependent acquisition of astrocytic differentiation capacity of NSCs. A CpG sequence of the STAT3 binding site (5′-TTCCGAGAA-3′) in the promoter of a typical astrocyte gene, *glial fibrillary acidic protein* (*GFAP*), is highly methylated at E11 when NSCs cannot differentiate into astrocytes even if stimulated with astrocyte-inducing cytokines such as LIF, and later becomes demethylated and thus allows transcription-factor binding at E14, when NSCs acquire the potential to differentiate into astrocytes [12]. Moreover, DNA demethylation which occurs in the promoter of another astrocyte gene, *S-100 calcium-binding protein beta chain* (*S100β*), between E11 and E14 is associated with decreased binding there of the transcriptional repressor methyl CpG-binding protein 2 (MeCP2) [12], suggesting that dissociation of methyl CpG-binding domain containing (MBD) protein family members from DNA derepresses a set of astrocyte genes in NSCs. In addition to *GFAP* and *S100β*, it has been shown that many other astrocyte genes synchronously become DNA demethylated, which enables NSCs to acquire the epigenetic ability to differentiate into astrocytes [14]. When E11 NSCs, which do not yet have the ability to differentiate into astrocytes, are cultured for several days *in vitro*, they acquire this ability, suggesting that early NSCs and their derivatives in culture contain the necessary components for the acquisition of astrocytic differentiation capacity by NSCs. E11 mouse cortical NSCs can produce *GFAP*-positive astrocytes when cultured for 4 to 5 days *in vitro* (DIV) with LIF stimulation. Interestingly, E11 NSCs prepared from NSC-specific maintenance DNA methyltransferase DNMT1 cKO mice could produce astrocytes much faster, after just 2 DIV with LIF stimulation. Furthermore, forced expression of DNMT1 inhibited astrocytic differentiation with LIF stimulation in E11 DNMT1 cKO NSCs cultured 5 DIV [15]. These *in vitro* experiments suggested that dissociation of DNMT1 occurs gradually at astrocytic gene promoters in NSCs as they acquire the epigenetic ability to undergo astrocytic differentiation. Such selective DNMT1 dissociation from astrocytic gene promoters is induced by cell-extrinsic stimulation. We have previously shown that activation of Notch signaling in E11 NSCs by direct cell–cell contact with neighboring immature neurons expressing Notch ligands leads to dissociation of DNMT1 from astrocytic gene promoters, resulting in their demethylation [11,16].

Although DNA methylation plays a critical role in regulating the expression of astrocytic genes, demethylation of the promoter is not sufficient for the genes to become fully competent to be

transcribed. In addition to DNA and histone modifications, chromatin/nucleosome remodeling at astrocytic gene promoters is also important for the acquisition of astrocytic differentiation capacity by NSCs. This is supported by the fact that in embryonic stem cells (ESCs) deficient for three DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b), the H3K9 methyltransferase euchromatic histone-lysine *N*-methyltransferase 2 gene (*Ehmt*, also called *G9a*), or *Eed*, LIF cannot induce *GFAP* expression, despite the fact that DNA methylation, H3K9 trimethylation (H3K9me3), and H3K27me3 in the promoter are abolished [17]. However, micrococcal nuclease digestion experiments showed that the chromatin state of the *GFAP* promoter is loosened in E14 NSCs, which express *GFAP* in response to LIF, but not in cells such as ESCs and E11 NSCs, which cannot express *GFAP* even if stimulated by LIF [17]. This suggests that unknown chromatin remodeling factors are involved in nucleosome remodeling at the *GFAP* locus. The candidate factors include members of the SWItch/Sucrose NonFermentable (SWI/SNF) family of chromatin-remodeling complexes that alter DNA-histone contacts in an ATP-dependent manner [18]. Mammalian SWI/SNF complexes contain one of two catalytic ATPase subunits: Brahma or Brahma-related gene 1 (*Brg1*, also called *Snf2β*). *Brg1* has been suggested to be essential for astrocytic gene expression, because, after NSC-specific deletion of *Brg1* in the mouse, E15 NSCs did not show the capacity of astrocytic differentiation even in the presence of LIF [19].

After the acquisition of astrocytic differentiation capacity by NSCs, a precise histone modification status maintains the undifferentiated status of these NSCs to prevent astrocytic differentiation. VPA treatment of E14 NSCs further enhances the LIF-mediated astrocytic differentiation partly via histone acetylation around the STAT3 binding site of the *GFAP* promoter [11], suggesting that histone hypoacetylation has a role in preventing NSCs from undergoing astrocytic differentiation. Similarly to HDACs, *Ezh2* is expressed in proliferating embryonic NSCs and subsequently suppressed during differentiation into astrocytes as well as neurons [3,20]. Conversely, ectopic expression of *Ezh2* in E16 NSCs sustained high H3K27me3 levels at the *GFAP* promoter and resulted in the inhibition of astrocytic differentiation in a PRC2-dependent manner [21]. Precise levels of chromatin proteins also maintain the undifferentiated status of multipotent NSCs. Nuclear receptor corepressor gene (*N-CoR*), which is highly expressed in proliferating NSCs in the VZ of the embryonic brain [22], is also important for inhibiting spontaneous astrocytic differentiation of multipotent NSCs through forming a complex with HDACs to maintain the repressed status of Notch downstream genes until NSCs receive cell-extrinsic Notch stimulation [23]. Astrocytic differentiation of embryonic NSCs upon ciliary neurotrophic factor (CNTF) stimulation results in translocation of *N-CoR* from the nucleus to the cytoplasm to be degraded. NSCs isolated from E13 *N-CoR*-deficient mouse brains spontaneously differentiate into astrocytes even when cultured in proliferation medium. This cell population shows markedly reduced neuronal differentiation capacity even when cultured in neuronal-differentiation medium. *N-CoR*-deficient mice develop normally until E12, but show a decrease in the size of specific brain areas and aberrantly upregulated GFAP expression at E14, and die before E16 [22]. Conversely, forced expression of *N-CoR* in NSCs represses *GFAP* as a result of direct binding of *N-CoR* and compromises the induction of astrocytic differentiation by CNTF [24]. A class III HDAC, sirtuin 1 (Sirt1), also contributes to the astrocytic differentiation of NSCs via the suppression of neuronal genes. Chemically induced Sirt1 activation in NSCs isolated from the E17 cortex suppressed their proliferation and directed them toward the astrocytic lineage at the expense of the neuronal lineage [25]. In that report, it was suggested that activated Sirt1–HES1 complex is recruited to the promoter of a known HES1 target, a proneural bHLH transcription factor achaete–scute complex homolog 1 (*ASCL1*; also called *Mash1*) gene, leading to the downregulation of H3K9 acetylation in the promoter [25].

Epigenetic regulation of postnatal oligodendrocyte differentiation and adult neurogenesis

We do not yet know whether all NSCs acquire multipotency to produce not only neurons and astrocytes but also oligodendrocytes in the early postnatal cerebral cortex. Regarding oligodendrocyte precursor (OPC) markers, cells that were positive for a myelin proteolipid protein (PLP) isoform, neuronal membrane glycoprotein M6-b-like (Dm-20), were first detected at E9 in the ventricular germinal layer of the laterobasal plate of the diencephalon during mouse brain development. By E14, Dm-20-positive cells had largely disappeared from the diencephalic ventricular germinal layer and had colonized the ventral mantle layer at the posterior part of the basal diencephalon [26]. After birth, the activation of sonic hedgehog (Shh) signaling promotes differentiation of NSCs to OPCs [27]. An appropriate level of histone acetylation is important for maintaining the NSC capacity that allows commitment to OPCs, and it was suggested that a decrease in histone acetylation triggers this commitment. Indeed, HDAC inhibition in OPCs by treatment with pharmacological inhibitors caused these committed progenitors to revert to multipotent cells characterized by expression of an NSC marker, SRY-box containing gene 2 (*Sox2*) [28]. Another study showed that treatment of OPCs from early postnatal rodent brain with a class I and II HDAC inhibitor (trichostatin A, TSA) inhibits myelination, and even enhances astrocytic and neuronal differentiation both *in vitro* and *in vivo* [16]. Such HDAC inhibitor-treated OPCs do not resume myelination at all even after withdrawal of HDAC inhibitors [16]. Involvement of PcG proteins in the transition from NSC to OPC has also been suggested. Overexpression of *Ezh2* in mouse NSCs increased the number of oligodendrocytes at the expense of astrocytes, whereas silencing of *Ezh2* yielded the opposite results [29]. The histone H2A E3 monoubiquitin ligase ring finger protein 1B gene (Ring1B), a member of the PRC1 complex, seems to be essential for oligodendrocyte differentiation. In E18 NSCs, *Ring1B* deletion through Cre-mediated recombination *in vitro* selectively resulted in a decreased number of oligodendrocytes, but the numbers of neurons and astrocytes were not affected [30].

After cells are committed to OPCs, mitogen withdrawal induces oligodendrocytic differentiation and myelination *in vitro* [16]. It should be noted that during oligodendrocytic differentiation of OPCs, histone acetylation levels are globally decreased [16], suggesting that histone deacetylation is important not only for NSC commitment to OPCs but also for OPC differentiation into mature oligodendrocytes. In fact, HDAC1 is highly expressed in the corpus callosum, where oligodendrocytic differentiation is frequently seen [31]. HDAC1 and 2 contribute to this genome-wide histone deacetylation and suppress the Wnt signaling pathway, leading to promotion of the expression of the inhibitor of differentiation 2 gene (*Id2*) and *Id4*, which inhibit myelin gene expression [30]. In support of this HDAC involvement in the suppression of inhibitory genes during oligodendrocyte differentiation, early postnatal OPCs isolated from oligodendrocyte-specific HDAC1 and 2 combined cKO mutant mice showed impaired oligodendrocytic differentiation [30]. Among the genes downregulated by histone deacetylation during oligodendrocyte differentiation, over 30% have the consensus sequence 5′-NNCCATNN-3′, a common binding motif of a zinc-finger protein transcription factor, Yin Yang 1 (YY1), in their flanking sequences. Oligodendrocyte lineage-specific *YY1* cKO mice appear to be normal at birth but develop shaking, tremor, and head wobbling symptoms, and show defective myelination in the P18 spinal cord [30]. YY1 recruits HDAC1 to the promoter regions of *Id4* and transcription factor 4 (whose products inhibit the expression of oligodendrocytic differentiation-associated genes), thereby decreasing their expression levels due to transcriptional repression during oligodendrocytic differentiation of early postnatal OPCs [30]. A class IV HDAC, HDAC11, is also highly expressed during myelination and modulates

deacetylation of H3K9/K14 on myelin genes during differentiation of OPCs. Increasing the expression level of HDAC11 results in its recruitment to the *myelin basic protein* and *Plp* genes. Conversely, an siRNA-induced decrease in the expression of *HDAC11* increased acetylated H3K9/K14 and led to reduced myelin gene expression [30].

In adult mice, multipotent NSCs continue to exist in restricted brain areas such as the SVZ and DG. Maintenance and differentiation of these particular NSCs are also regulated physiologically by epigenetic factors. MBD1 is relatively highly expressed in adult brain neurons and regulates the expression of basic fibroblast growth factor 2 (FGF2), which is used to expand NSCs *in vitro*. NSCs from adult *MBD1* knockout mice showed hypomethylation of the promoter region of *FGF2* associated with increased expression of *FGF2*, and a great reduction of neuronal differentiation capacity [32]. DNA methylation inhibitor treatment of NSCs also caused similar effects.

A recently discovered form of DNA modification, 5′-hydroxymethylcytosine (5hmC), which can be a target of the base excision repair, is associated with an active DNA demethylation process. Ten-eleven translocation 1 (TET1), 2, and 3 convert 5′-methylcytosine (5mC) to 5hmC in an Fe (II)- and α-ketoglutarate-dependent manner [33]. During embryonic brain development, high levels of 5hmC are detected. TET1 or *2* single knockout mice appear to undergo normal embryonic and early postnatal development, although TET1 and 2 are normally highly expressed in adult brain and neuronal layers of the embryonic cortex, respectively [33]. Recently, more detailed analysis has revealed that TET1-deficiency impairs self-renewal of NSCs and causes aberrant DNA methylation, resulting in a significant decrease of adult neurogenesis [34]. In fact, in the adult mouse DG, TET1 was shown to be involved in neuronal activity-induced DNA demethylation of promoter regions of proliferation-related *FGF1b* and neuronal maturation-related brain-derived neurotrophic factor (BDNF), followed by their transcriptional upregulation [33].

Combination of *trans*-acting epigenetic modifiers involved in epigenome formation

Figure 4.2 summarizes the changes in the enzymatic activity of epigenetic modifiers that regulate the opening and closing of chromatin conformation during mouse cortical development described above. Roughly by E11, a fraction of cells acquire neuronal differentiation capacities, probably in parallel with decreasing H3K27me3 levels. Subsequently, the H3K27me3 level further decreases, followed by early-born neuron production. Concurrently, histone hyperacetylation also promotes neuronal differentiation. Although the mechanism of transition from DL to UL neuron production is still unknown, global histone hyperacetylation caused by pharmacological HDAC inhibition results in increased production of UL neurons and decreased production of DL neurons. In E14 NSCs, a genome-wide decrease in H3K27me3 levels and Notch-induced DNA demethylation occur, especially at astrocytic gene promoters, to preset the cells for acquiring the capacity of astrocytic differentiation. Around E16-P0, at the time of astrocytic differentiation, histone acetylation occurs in the promoters of astrocyte genes. After birth, NSCs mainly produce astrocytes and oligodendrocytes. Although modifications usually associated with open chromatin structure are observed at the onset of NSC differentiation into both neurons and astrocytes, oligodendrocyte differentiation is associated with the upregulation of epigenetic modifiers for closed chromatin formation. *Ezh2* is highly expressed in early postnatal NSCs and OPCs. During oligodendrocyte differentiation, genome-wide histone deacetylation takes place. However, when histone

acetylation level is compared among adult NSCs and their differentiated cells, it is quite different from that in developing stages depicted in Figure 4.2: It is highest in adult NSCs and adult NSC-derived neurons, followed by oligodendrocytes, and lowest in astrocytes [35]. It should be noted that the neural cell type specification after changes in expression of epigenetic modifiers does not seem to be attributable to the gain or loss of genome-wide epigenetic marks. Rather, a single neural cell has a mechanism to limit the effect of the change in epigenetic modifier expression on a set of genes in a cell-context-dependent manner. In this context, it is worthwhile discussing [1] combinations of epigenetic modifiers that can increase the sequence-specific recognition of a set of genes, and [2] the potential of non-coding RNAs (ncRNAs) to efficiently guide epigenetic modifiers to build up a cell lineage-specific epigenome.

The combination of epigenetic modifiers is important for their target gene selection. For example, in early NSCs, a decrease of *Ezh2* activity is essential for generating early-born neurons, whereas postnatal NSCs have neuronal differentiation capacity in spite of the fact that *Ezh2* expression in NSCs can be observed again after birth [36]. Therefore, there should be mechanisms for neuronal genes to escape from H3K27me3-mediated polycomb repression. Indeed, DNMT3a causes gene-body DNA methylation, which functionally antagonizes polycomb repression, of distal-less homeobox 2 (*Dlx2*), an interneuron-associated gene, and expression of *Dlx2* in postnatal NSCs is thereby kept active. During brain development, DNMT3a and DNMT3b are expressed in a complementary fashion [37]. DNMT3b begins to be expressed in early embryonic cells and neural progenitor cells during neurogenesis prior to DNMT3a. DNMT3a is predominantly expressed at later developmental stages, such as just after differentiation from neural progenitor cells [37]. In postnatal NSCs derived from DNMT3a knockout mice, neurogenesis is severely inhibited. Intriguingly, in postnatal NSCs, the high DNA methylation level of the *Dlx2* gene body locus contributes to maintenance of a transcriptionally active chromatin state by preventing the locus from PRC2 complex association [36]. In parallel with *Dlx2* gene body methylation by DNMT3a, mixed-lineage leukemia 1 (Mll1), an H3K4-specific methyltransferase, functions to keep an active chromatin state at the promoter of *Dlx2* in mouse NSCs. Mll1 deletion in mouse results in *Dlx2*-repression and then impairs postnatal neuronal differentiation without a significant change in the NSC proliferation rate. Lack of Mll1 increased the methylation level of H3K27 specifically at the *Dlx2* promoter, preventing the association of transcriptional activator complexes [38], which include an H3K27-specific histone demethylase, a member of the Jumonji C domain-containing histone demethylase (JMJD) family [38]. These facts indicate that both transient transcriptional upregulation of *Dnmt3a* and formation of an active chromatin complex including Mll1 and JMJD on *Dlx2* are critical for protecting against methylation of H3K27, leading to maintenance of the neuronal differentiation capacity of postnatal NSCs. The importance of the combination of epigenetic modifiers is also suggested by contradictory effects between chemical inhibition and gene deletion of HDACs. Treatment of embryonic cortical NSCs with a class I and II HDAC inhibitor, TSA, promotes neuronal differentiation and inhibits gliogenic differentiation [2], suggesting that decreasing HDAC activity is important for neurogenesis. On the other hand, in mouse embryonic NSCs, HDAC1/2 combined deletion not only inhibits cell-cycle progression but also increases cell death, resulting in the impairment of proper neurogenesis [39]. It seems likely that the residual activity of HDACs in NSCs treated with pharmacological inhibitors is sufficient for normal cell-cycle progression and survival, while it promotes neuronal differentiation of NSCs. Taken together, these findings suggest that small but significant changes in epigenetic modifier activity may determine the identities of NSCs and their differentiated descendants.

The responses of cells to changes in *trans*-acting epigenetic modifier activity are considered to be determined by the status of their respective epigenomes. However, how the response is determined

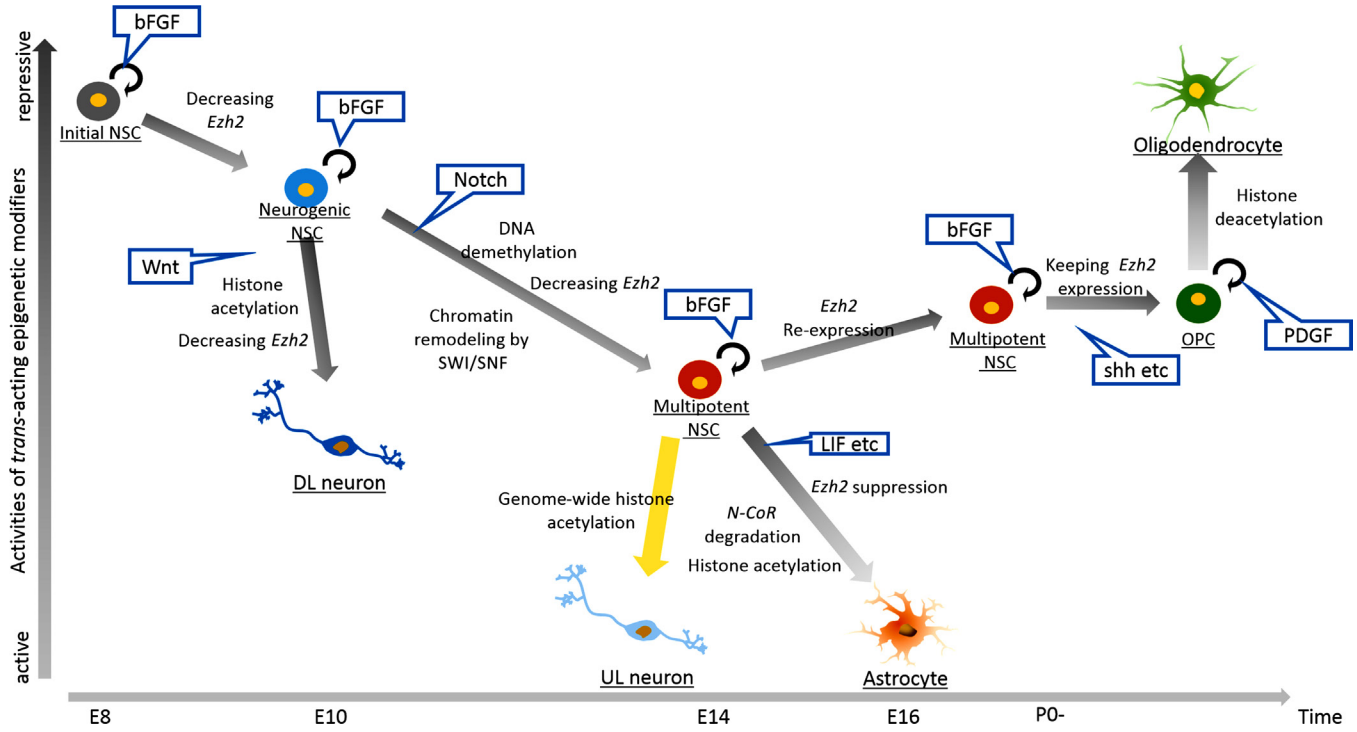

FIGURE 4.2

Summary of expression changes of epigenetic modifiers during mouse cortical development. In early cortical development, NSCs acquire neuronal differentiation capacity, probably following the decrease of *Ezh2* expression. These neurogenic NSCs produce neurons in response to Wnt stimulation. The neurogenic NSCs switch to producing UL neurons through local histone acetylation changes. In parallel, Notch signals from newly born immature neurons are received by the neighboring NSCs. Subsequently, these NSCs acquire astrocytic differentiation capacity through DNA demethylation of astrocytic gene promoters. In this step, SWI/SNF chromatin remodeling complex may help chromatin remodeling of astrocytic gene promoters. Subsequently, resulting multipotent NSCs produce astrocytes in response to LIF and other cytokine stimulations through dissociation of *N-CoR* from astrocytic genes and its degradation. During oligodendrocytic differentiation after birth, genome-wide histone deacetylation occurs. In this figure, the OPCs generated before birth are not depicted.

remains largely unknown. In this context, recent studies have suggested that ncRNAs function as molecules with variable sequence specificities that can link a specific pattern of epigenomes with *trans*-acting epigenetic modifiers. In general, miRNAs, a class of small double-stranded RNA (dsRNA), recognize the 3′UTR sequence of target mRNAs and inhibit their translation or promote their degradation. For example, miR-124 has three major direct targets—neuronal gene *Dlx2*, Notch ligand *jagged-1*, and astrocyte gene *Sox9*—and reduces their protein products [40]. Another reported function of small dsRNA is to regulate chromatin status by converting a repressor-type complex on neuronal genes to activator-type to induce gene expression. In NSCs, a transcriptional repressor, neuronal restricted silencing factor/RE-1 silencing transcription factor (NRSF/REST), recognizes and binds to the consensus sequence named neuron restrictive silencer element/RE-1 (NRSE/RE1) commonly found in a large fraction of neuronal genes. A small dsRNA complementary to this particular sequence, thus named NRSE dsRNA, associates with NRSF/REST to de-repress or even activate NRSE/RE1-containing genes during an early step of neuronal differentiation of adult NSCs [41].

In contrast, as exemplified by *utNgn1* described above, lncRNAs (>200 nt) function at the level of epigenetic regulation via different mechanisms from that of the small dsRNAs. A significant proportion of lncRNAs exhibit tissue-specific expression patterns [42], and they can recruit epigenetic modifiers to specific loci and contribute to cell-fate determination via local epigenetic regulations. For example, an lncRNA, rhabdomyosarcoma 2-associated transcript (*RMST*), is specifically expressed in the mammalian brain and is strongly upregulated upon retinoic acid (RA)-induced neuronal differentiation of human NSCs. *RMST* enhances *Sox2*-targeting to neuronal genes such as *Dlx1* and *ASCL1* via complex formation with *Sox2*. This finding was supported by the facts that the ability of *Sox2* to bind to these neuronal gene promoters is lost and RA-induced neuronal differentiation fails in *RMST* knockout NSCs [43]. Of interest, most brain-expressed lncRNAs are observed to be preferentially located adjacent to protein-coding genes that are highly expressed in the brain during mouse development [44]. Another type of lncRNA named antisense promoter-associated ncRNA (pancRNA) has been shown to behave as a sequence-specific epigenetic modifier [45], although the underlying mechanisms are not completely understood. Each pancRNA shares its promoter sequence with its respective adjacent region transcribed as mRNA and is transcribed in the opposite direction to that of the mRNA. PancRNAs are associated with several active chromatin marks at the promoters of two neural genes, *neurofilament light polypeptide* and *vimentin*, in rat PC12 cells but not in a fibroblastic cell line. Forced expression of these pancRNAs induced sequence-specific active DNA demethylation in the promoter region. Conversely, a reduction of pancRNA levels increased DNA methylation of the promoter [45]. Therefore, it could be expected that such pancRNAs are produced during the cell lineage commitment for initiation and/or maintenance of cell-type-specific epigenomic profile. However, most functions of lncRNAs have thus far remained unknown.

Importance of focusing on epigenetics in neuronal functions

Epigenetic mechanisms that mediate precisely orchestrated gene transcriptional regulations are fundamental to the functions of neurons as well as the differentiation of NSCs. Although epigenetic modifications are considered to be hard to remove once established, it is becoming apparent that they are actually more plastic in response to intrinsic and extrinsic cues than previously thought. For example, DNA methylation status fluctuates according to the cellular environment. So far, many studies have identified the enzymes responsible for the writing of several types of histone modifications

and for their erasure. In addition to DNA and histone modifications, an increasing body of research has shown that the nucleosome positioning and the histone turnover are regulated in a cell-cycle-independent manner [46]. Such epigenetic plasticity is defined as how much the epigenetic status can change to another status at the genomic locus. The sensitivity of a certain genomic locus to epigenetic changes can differ from that of the adjacent loci in a cell-type-specific manner. In the following section, we describe the impact of epigenetic regulation on synaptic plasticity, memory and learning, and the occurrence of epigenetic changes depending on the neuronal activity. Then, by considering the epigenomic profile unique to neurons, we discuss the relatively low epigenetic plasticity of neurons possibly underlying their robustness against perturbation of their intracellular environment by exogenous *trans*-factors.

Impact of epigenetic regulation on synaptic plasticity, learning, and memory

The causal association between epigenetic regulation and neuronal function has been proven by the combined findings of a large body of research on learning and memory. For example, epigenetic regulation seems to modulate synaptic activity by altering the expression of synaptic plasticity-related genes. In the adult mouse hippocampus, Reelin and BDNF proteins are implicated in the induction of synaptic plasticity [47]. It is known that the dynamics of DNA methylation status at the promoter regions of the Reelin gene, *Reln*, and of *BDNF* can regulate their transcription, thereby affecting synaptic plasticity. Epigenetic regulation affects long-term potentiation (LTP), which is a long-lasting enhancement of signal transmission between two neurons and represents a physiological form of synaptic plasticity underlying learning and memory formation. In mouse hippocampal slice cultures, DNMT inhibitors could block LTP but did not affect synaptic transmission [47]. Combined deletion of *Dnmt1* and *Dnmt3a* specifically in mouse forebrain excitatory neurons led to abnormal LTP in the hippocampal CA1 region together with deficits in memory consolidation, but, interestingly, no impairment in memory acquisition [48]. In patients with schizophrenia or bipolar disorder, *Dnmt1* mRNA and its protein expression are preferentially increased in cortical interneurons, implying the involvement of DNA methylation in psychiatric disorders [49]. Thus, the proper DNA methylation profile is probably indispensable for normal synaptic plasticity, memory consolidation, and mental status.

After methylation or demethylation of DNA, MBD proteins could translate DNA methylation profiles to many kinds of biological processes implicated in neuronal functions. For instance, MeCP2 is highly expressed in neurons and functions in chromatin remodeling and transcriptional regulation according to the DNA methylation profile of the genes. In humans, loss-of-function mutations of *MeCP2* cause Rett syndrome, an X-linked neurodevelopmental disorder [50]. MeCP2 binds to methylated CpG sites in the genome and recruits transcriptional repressors or activators such as Sin3a or Creb1 to induce repression or activation of the target gene transcription, respectively [8,10]. It has been reported that MeCP2 bound to methylated CpGs becomes phosphorylated and dissociates from DNA in a neuronal activity-dependent manner [51]. Transgenic mice in which *MeCP2* is mutated at the neuronal-activity-induced phosphorylation site have been generated. These transgenic mice showed enhanced LTP and increased excitatory synaptogenesis in the hippocampus [52]. Taken together, these studies suggest that MeCP2 plays a critical role in translation of the DNA methylation profile in neurons to the gene expression profiles influencing synaptic plasticity and synaptogenesis.

Several types of histone modification also participate in neuronal function. For example, it has been reported that several psychiatric diseases are associated with histone modifications [50]. One such disease is Rubinstein–Taybi syndrome, a well-defined disease of multiple congenital anomalies including mental retardation and skeletal abnormalities. Mutations in two HATs (CBP and p300) commonly cause this syndrome [50]. Alarcón and colleagues showed that LTP and long-term memory were impaired in the *Cbp* mutant mouse [53]. The LTP deficit caused by the mutation in *CBP* was abrogated by enhancing the expression level of CREB-downstream target genes such as the *BDNF*, *dynorphin*, and c-*fos* genes [53]. CREB–CBP complex positively regulates the transcription of nuclear receptor genes *Nr4a1* and *Nr4a2*, which are involved in neuronal function [54]. Moreover, CBP is also known to play a role in the transition of short-term to long-term memory. Similarly to *CBP* mutant mice, transgenic mice expressing a loss-of-function mutant of either the *p300* or *p300/CBP-associated factor* gene exhibit deficits in long-term memory formation [55,56].

As mentioned earlier, the histone acetylation profile is defined by the balance between the activities of HATs and HDACs. Vecsey and colleagues showed that inhibition of HDACs with TSA could enhance hippocampal synaptic plasticity and hippocampus-dependent memory, whereas some HDACs are known to negatively regulate LTP via transcriptional repression of genes, such as *Nr4a1* and *Nr4a2*, in the mouse hippocampus [54]. Increase of the histone acetylation levels by treatment with another HDAC inhibitor, sodium butyrate, could also induce sprouting of dendrites, an increased number of synapses, recovery of learning, and re-establishment of access to long-term memories after significant brain atrophy and neuronal loss had already occurred [57]. Neuron-specific overexpression of *HDAC2* decreased the dendritic spine density and number of synapses, and abolished LTP in mouse hippocampal neurons [58]. On the other hand, neuron-specific *HDAC1*-overexpression in mice did not appear to phenocopy the *HDAC2*-overexpression in terms of the synaptogenesis and synaptic plasticity. Moreover, mice overexpressing *HDAC2*, but not *HDAC1*, showed impaired memory formation. *HDAC3* is reported to be a negative regulator of long-term memory formation. Deletion of *HDAC3* in the hippocampus was accompanied by increases in the expression levels of *Nr4a2* and c-*fos*, leading to the enhancement of long-term memory for object location [59]. These results suggest that *HDAC2* and *HDAC3*, but not *HDAC1*, decrease the synaptic plasticity represented by LTP attenuation and inhibit memory formation.

The regulation of histone methylation also underlies neuronal functions (e.g., cognitive functions such as learning and memory, and psychiatric conditions). As described above, H3K9 methylation is a well-known histone modification associated with closed chromatin. In mouse postnatal neurons, the loss of a histone methyltransferase complex composed of G9a-like protein (GLP) and G9a, which catalyzes H3K9me2 methylation, leads to complicated behavioral abnormalities, including defects in learning, motivation, and environmental adaptation [60]. It has also been reported that the expression of GLP and G9a is repressed by repeated cocaine administration, and that such repression induces upregulation of the expression of several genes, including *BDNF*, an increase of the dendritic spine plasticity of neurons in the nucleus accumbens, and the development of cocaine addiction [61]. Another type of histone methylation at H3K27, which is associated with transcriptional repression, is also likely to be involved in depression through the repression of *BDNF* [50]. In contrast, histone methylation at H3K4 plays different roles. The analysis of H3K4 methyltransferase *Mll* heterozygous mutant mice has revealed that H3K4 methylation is necessary for the proper long-term consolidation of contextual fear memories [62]. Consistently, Mll1-mediated H3K4 methylation at the promoter of the GABAergic gene *GAD1* has been suggested to play a role in the prefrontal cortex dysfunction in schizophrenia [63].

Plasticity of epigenetic profile in neurons in response to extrinsic stimuli

Dynamic changes of neuronal gene expression in response to extrinsic stimuli are mediated by epigenetic regulation. In postmitotic neurons, there are genomic regions where the epigenetic status can be dynamically altered by neuronal activity. A growing number of reports have suggested that these changes can potentially influence synaptic plasticity, and the formation and maintenance of memory (Figure 4.3).

In postmitotic neurons, there is increasing evidence supporting the existence of replication-independent active DNA methylation and demethylation. The dynamics of DNA methylation status reflects the balance of two sets of reactions: DNA methylation and DNA demethylation. The maintenance DNA methyltransferase DNMT1 and *de novo* DNA methyltransferase DNMT3A are expressed in postmitotic neurons. In contrast to the ubiquitously expressed *DNMT1*, *DNMT3a* and *DNMT3b* play different roles in neural development and function in stage- and cell type-specific manners. Recent studies have suggested that several *trans*-factors participate in active DNA demethylation through the generation of 5hmC. Indeed, the highest levels of 5hmC are detected in the brain among the tissues examined [33]. Although the precise mechanism leading to DNA demethylation in neurons remains controversial, TET proteins are suggested to be key molecules in the DNA demethylation mechanism,

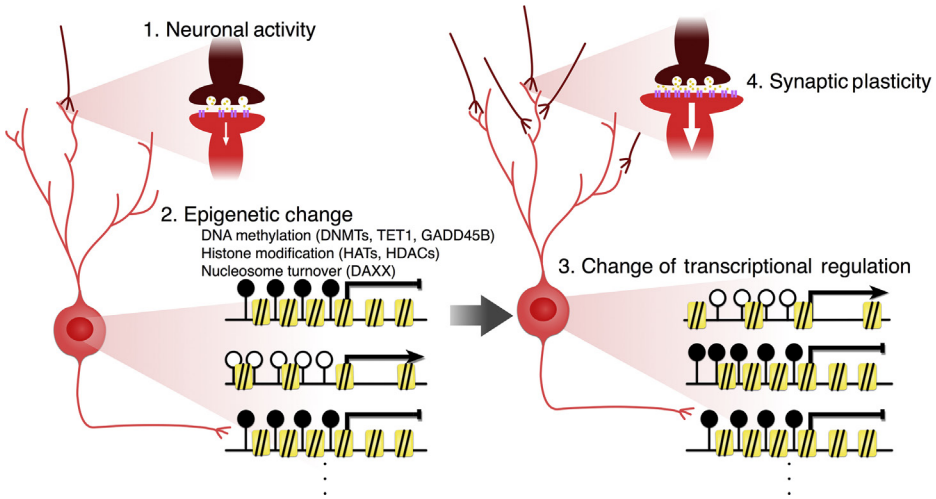

FIGURE 4.3

Epigenetic changes observed in postmitotic neurons. Neuronal activity can induce several changes of biological processes, such as the expression levels of epigenetic modifiers, leading to global and local changes of the epigenetic status. These epigenetic changes alter transcriptional regulation at several gene loci (e.g., *BDNF*), followed by enhancement of synaptic plasticity. Filled and open lollipops denote methylated and unmethylated cytosine, respectively. Arrows and hammers indicate expression and repression of downstream genes, respectively.

as described above. One of the TET-related active DNA demethylation mechanisms reported by Guo and colleagues involves the activation-induced deaminase (AID)/apolipoprotein B mRNA-editing enzyme complex (APOBEC) family of cytidine deaminases [33]. They proposed that 5hmCs produced by oxidation of 5mCs by TET proteins are deaminated by AID/APOBEC deaminases into 5′-hydroxyuracils (5hmUs) followed by base excision repair (BER) by a 5hmU glycosylase, such as thymine DNA glycosylase or single-strand-selective monofunctional uracil–DNA glycosylase 1, and eventually replaced with unmethylated cytosines. In fact, *TET1* and *APOBEC1* mRNAs are substantially expressed and promote brain-area-specific neuronal activity-induced DNA demethylation in the mature neurons of the adult mouse DG [33]. On the other hand, other reports propose a different mechanism in which TET proteins catalyze conversion from 5mC to 5′-carboxylcytosine (5caC), followed by BER [33]. It has been reported that *TET3* mRNA encodes a predominant form of TET proteins in the mouse brain and is expressed in several brain areas and neural cell types, such as embryonic cortical NSCs and neurons, and adult cerebral cortex, cerebellum and hippocampus [33]. In agreement with the variable expression of TET proteins, 5hmC is found at variable levels in mammalian cells. It has been reported that 5hmC is present at about 40% of the total level of 5mC in Purkinje neurons [33]. In cerebral cortex neurons, 5hmC is associated preferentially with gene bodies of activated neuronal function-related genes [33].

Table 4.1 summarizes the influences of neuronal activity on the epigenetic status. In primary cortical neurons, membrane depolarization can cause a temporary reduction in the mRNA and protein expression of both DNMT1 and DNMT3A. The downregulation of DNMT activities could induce the upregulation of *BDNF* expression. This depolarization of primary cortical neurons was found to induce DNA demethylation at specific sets of *BDNF* promoters followed by the release of MeCP2, leading to the expression of *BDNF* isoforms [47]. The DNA methylation profile in neurons may be defined by a local balance of DNA methylation and DNA demethylation activities at a set of gene loci. Based on this premise, it is conceivable that a decrease of DNA methylation activity would tip this balance toward DNA demethylation. Similar DNA demethylation by inhibition of DNMT was reported in adult hippocampal neurons [76]. Nelson and colleagues showed that neuronal activity-dependent DNA demethylation was mimicked by DNMT inhibitors and blocked by an *N*-methyl-D-aspartate (NMDA) receptor antagonist, respectively, supporting the idea that a decrease of DNMT activity by neurotransmitters is critical for the induction of DNA demethylation. In the mouse frontal cortex and hippocampus, stimulation with an agonist of metabotropic glutamate receptors (GRM2 and 3) decreased the DNA methylation level at the specific promoter regions of *Reln*, *BDNF*, and a schizophrenia and bipolar disorder-related gene, *glutamate decarboxylase-67* (*GAD67*) [64]. In the model of synchronous activation of mature neurons in the DG of the adult mouse brain by electroconvulsive treatment *in vivo*, active DNA demethylation of *BDNF* exon IX and *FGF1b* promoter regions also occurs with the help of Gadd45b, another protein related to DNA demethylation involving the DNA repair process [47]. Taken together, these findings indicate that the balance of DNA methylation and demethylation activities in the active turnover of DNA methylation could depend on neuronal activity, leading to changes of the transcriptional regulation of genes associated with neuronal functions.

Histone modifications as well as DNA methylation dynamically change in response to neuronal activity. For example, in slice cultures of the adult mouse CA1 hippocampus area, activation of NMDA receptors increased the genome-wide level of acetylated histone H3 through the extracellular signal-regulated kinase (ERK) signaling cascade [68]. Another example is that the neuronal activity-triggered influx of Ca^{2+} activates Ca^{2+}/calmodulin-dependent kinase (CaMK)-IV, followed by the phosphorylation of CBP, one of the major histone acetyltransferases. The phosphorylated CBP is subsequently recruited to the

Table 4.1 Extrinsic Stimulus-Induced Epigenetic Changes

Extrinsic Stimulus	Epigenetic Modification	Target Gene Loci	Ref.
Neuronal activity	DNA demethylation	*BDNF* *Reln* *GAD67* *FGF1b*	Day and Sweatt [47] Matrisciano et al. [64] Matrisciano et al. [64] Day and Sweatt [47]
	Histone acetylation	*Nr4a1* [54] *Nr4a2* [54] *BDNF* [65]	Vecsey et al. [54] Vecsey et al. [54] Lubin et al. [65]
	Phosphorylation of H3S10	Global	Crosio et al. [66]
	Replacement of H3.1 by H3.3	*BDNF* *c-fos* *Npas4*	Michod et al. [67] Michod et al. [67] Michod et al. [67]
Contextual fear conditioning training	Histone acetylation	Global *BDNF*	Levenson et al. [68] Lubin et al. [65]
	DNA demethylation	*PP1* *BDNF* *Reln*	Miller and Sweatt [69] Miller and Sweatt [69] Miller and Sweatt [69]
	Phosphorylation of H3S10	Global [70]	Chwang et al. [70]
	DNA demethylation	*CaN* [71]	Miller et al. [71]
Eyeblink classical conditioning	Histone acetylation	Global *BDNF*	Fontán-Lozano et al. [72] Fontán-Lozano et al. [72]
Object recognition memory	Histone acetylation	Global *BDNF*	Fontán-Lozano et al. [72] Fontán-Lozano et al. [72]
Maternal behavior to offspring	DNA demethylation	*NR3C1*	Weaver et al. [73]
	Histone acetylation	*NR3C1*	Weaver et al. [73]
Childhood abuse	DNA methylation	*NR3C1*	McGowan et al. [74]
	DNA demethylation	*FKBP5*	Klengel et al. [75]

promoters and enhancers of CREB-regulated genes to change the chromatin configuration and induce gene expression [77]. It was also reported that membrane depolarization induces histone acetylation on the promoters and enhancers of CREB-regulated genes in a CaMK- and ERK-activity-dependent manner. The inhibition of histone deacetylation due to the phosphorylation-induced nuclear export of HDAC4 and HDAC5 further supports the hyperacetylated status of target genes as a result of neuronal activity-dependent CaMK activation mediated by the activation of NMDA receptors in hippocampal neurons [77]. This neuronal activity-induced global histone acetylation could affect the expression of many genes (*BDNF*, *Nr4a2*, etc.) simultaneously [54,65]. Regarding neuronal-activity-dependent histone modifications other than acetylation/deacetylation, much less information is available. For example, it is well known that phosphorylation of histone H3 at serine 10 (H3S10) is causally linked to chromosome condensation during both mitosis and meiosis, but not in cells that have already exited the cell cycle. Nonetheless, this modification can also be observed in postmitotic neurons. In adult mouse hippocampal neurons, stimulation with agonists for various neurotransmitter receptors, such as dopaminergic receptor, muscarinic acetylcholine receptor, and kainate glutamate receptor, rapidly induces the phosphorylation of

histone H3S10 on a genome-wide level. Phosphorylation of H3S10 is coupled to acetylation at the nearby H3K14 residue, leading to the transcriptional activation of genes [66].

The crosstalk between the dynamics of DNA methylation and histone modification in a neuronal activity-dependent manner is highly complicated. As already mentioned, the level of *BDNF* mRNA is upregulated by DNA demethylation and histone acetylation in a neuron-activity-dependent manner. However, the reverse is also true; that is, *BDNF* can affect the histone modification. In neurons, *BDNF* triggers nitric oxide synthesis and *S*-nitrosylation of HDAC2. This modification of HDAC2 induces its release from chromatin and results in an increase of the acetylation of histone H3 and H4 on specific gene promoters, leading to their transcriptional upregulation. Moreover, not only histone modification but also the components of nucleosomes are altered in response to neuronal activity. The histone chaperone death-domain associated protein (DAXX) is expressed in postmitotic neurons and localizes in the nucleus [67]. Neuronal activation induces the phosphorylation of DAXX, and phosphorylated DAXX loads H3.3, a histone variant of H3.1 that is linked with activity-dependent transcription, to the *BDNF*, c-*fos*, and *Npas4* loci, promoting the transcriptional activation of these three genes.

Social experiences are converted to sets of various neuronal stimuli, and therefore are somehow connected to complicated epigenetic changes (Table 4.1). Maternal behaviors toward offspring, such as pup licking and grooming and arched-back nursing, alter the DNA methylation level and acetylation of histone H3 of the glucocorticoid receptor gene (*NR3C1*) promoter in the rat hippocampus [73]. These epigenetic modifications directly affect the binding of transcriptional factor nerve growth factor-induced protein A, which is responsible for the transcription of *Gr*. Eyeblink classical conditioning, which is an associative-learning paradigm, and object recognition memory, which is a one-trial model of learning and memory, also increase the acetylation of histone H3 in the mouse hippocampus [72]. One of the experimental tests for monitoring memory—contextual fear conditioning training—activated the transcription of *Reln* through DNA demethylation at its promoter region in the mouse hippocampus [65,69]. Moreover, this training could also induce changes of histone modification—for example, a global increase of the acetylation and phosphorylation levels of histone H3 [68,70] and an increase of histone acetylation at the *BDNF* promoter IV [65]. However, in another genomic context, contextual fear conditioning training rapidly induced DNA hypermethylation at the promoter regions of the memory suppressor gene protein phosphatase 1 (*PP1*) and the transcriptional repression of *PP1* in the mouse hippocampus [69]. Such training-induced DNA hypermethylation is considered to be mediated by upregulation of DNMT1 expression in the mouse hippocampus, because treatment with DNMT1 inhibitor blocked memory formation [69]. Similarly, contextual fear conditioning induced DNA methylation of the calcineurin (CaN) promoter region followed by the downregulation of *CaN* expression in the mouse prefrontal cortex [71]. This downregulation of *CaN* could facilitate LTP, probably enhancing the maintenance of long-lasting memory formation. Childhood abuse also gene-dependently induces both DNA methylation and demethylation. It was reported that in the human hippocampus, childhood abuse induces abnormal DNA methylation at the NR3C1 promoter followed by a decrease of *NR3C1* mRNA persisting into adulthood [74]. In addition, another group showed that childhood abuse could affect the DNA methylation level at the FK506 binding protein 5 gene (*FKBP5*), an important negative regulator of *NR3C1* activity in terms of ligand binding [75]. In this context, it will be important to unveil the mechanisms inducing these two opposite epigenetic pathways by investigating whether these reactions occur in a single cell and what kinds of stimuli are responsible for these epigenetic changes.

The above-noted epigenetic changes are relatively transient and reversible. Considering the definition of epigenetics, we next need to highlight the different levels of epigenetic regulation that underlie the robustness of the neuronal identity after the cell lineage specification through processes described above.

The robust identity of neurons defined by the epigenetic status

Recently, Yamanaka and colleagues demonstrated that ectopic expression of *Oct3/4*, *Sox2*, *Klf4*, and c-*Myc* (OSKM) could reprogram differentiated fibroblasts to cells which show characteristics closely resembling those of ESCs, and therefore named such cells induced pluripotent stem cells (iPSCs) [78]. Since Yamanaka's study, numerous studies have shown that various types of somatic cells can acquire pluripotency upon the overexpression of OSKM. Of note, the ratio of successful reprogramming by OSKM varies depending on the cell type even if the genetic background is identical, strongly suggesting the relevance of epigenetic control [79]. Furthermore, the ectopic expression of OSKM is not sufficient to reprogram postmitotic neurons to iPSCs [80]. This robustness of neuronal identity may reflect neurons' epigenetic profile, including the accessibility of *trans*-factors to chromatin. Somatic cell type-specific genes generally become repressed by the formation of closed chromatin during the transition from differentiated cells to iPSCs. The pluripotency-associated genes, such as *Oct3/4* and *Nanog*, are conversely repressed by the closed chromatin status (H3K9me3 and methylated CpGs) in differentiated somatic cells; thus, ectopically expressed OSKM hardly bind to the endogenous promoters of their target genes. To overcome this robustness, the manipulation of epigenetic modifiers can strikingly affect the efficiency of reprogramming. For instance, depletion of H3K9 methylases (e.g., SUV39H1, 2) enhances the reprogramming of somatic cells to iPSCs, and overexpression of H3K9 demethylase (e.g., Lysine-specific demethylase 4B) also enhances the reprogramming [81]. In addition, H3K4 methylase (WD repeat domain 5, a key component of the trithorax group) and H3K27 methyltransferase (*Ezh2*, a key component of PRC2) are required for reprogramming to iPSCs [82]. Similarly, regarding DNA methylation, DNMT1, DNMT3A and 3B, and TET1 and 2 can affect the efficacy of reprogramming [82,83]. Moreover, many reports have shown that the association of OSKM with several epigenetic modifiers (e.g., Jumonji domain-containing protein 1 and DNMT3B) is crucial for the establishment and maintenance of the epigenomic status unique to iPSCs [84]. Therefore, reprogramming to iPSCs requires erasure of the epigenetic status unique to each cell type for rewriting to the epigenetic status unique to the pluripotent stem cell.

Mature neurons, which have exited from the cell cycle, have low efficiency of reprogramming to iPSCs with OSKM [80], as mentioned above. In this context, it is interesting to note that p53 suppression is crucial for efficient reprogramming of neurons to iPSCs. Although neurons and glial cells are both generated from NSCs, it is much easier to reprogram glial cells and NSCs into iPSCs compared with neurons [85,86]. The efficiency of transdifferentiation, as well as the reprogramming to iPSCs, also depends on the cell type. Therefore, the above findings would lead to the expectation that neurons may exhibit lower potency of transdifferentiation into different lineages compared with glial cells. Transdifferentiation is the conversion of a differentiated cell to alternative lineages, such as from fibroblasts to neurons [87]. Regarding glial cells, we have previously shown that activation of the JAK/STAT signaling pathway can induce transdifferentiation from oligodendrocytes into astrocytes [88]. Pericytes, a cell type implicated in the establishment and maintenance of the blood–brain barrier, isolated from human cerebral cortex were

also reported to be transdifferentiated into neurons by the ectopic co-expression of *Sox2* and *ASCL1* [89]. In addition, the ectopic expression of *Pax6* or *Ngn2* in astrocytes could induce their conversion to immature neurons [90]. Moreover, the plasticity of astrocytes was shown by their transdifferentiation into fully differentiated glutamatergic and GABAergic neurons by using *Ngn2* or *Dlx2*, respectively [90]. In contrast to glial cell transdifferentiation, there are few reports showing the transdifferentiation of neurons into different cell lineages. However, a recent study showed that overexpression of the FEZ family zinc finger 2 gene could induce the direct conversion of postmitotic callosal neurons into corticofugal neurons [91]. Nonetheless, there are no reports in which neurons have been converted to glial cells. Collectively, these facts support the notion that the robust epigenetic profile of neurons may underlie their extremely low capacity for cellular reprogramming.

Which molecular mechanisms contribute to the epigenetic robustness of neurons? One important clue is the fact that p53 inhibition together with OSKM enables reprogramming of postmitotic neurons into iPSCs [80]. *p53* is a well-known tumor suppressor gene which plays an important role in cellular proliferation. In fibroblasts, an increase of the cell proliferation rate by either inhibition of the p53/p21 pathway or overexpression of *Lin28* could accelerate conversion to iPSCs [92]. Likewise, knockdown of *p53* induced re-entry of postmitotic neurons into the cell cycle during the reprogramming to iPSCs [80]. It is thus likely that the cell-cycle-regulating machineries play a critical role in the reprogramming to iPSCs (Figure 4.4).

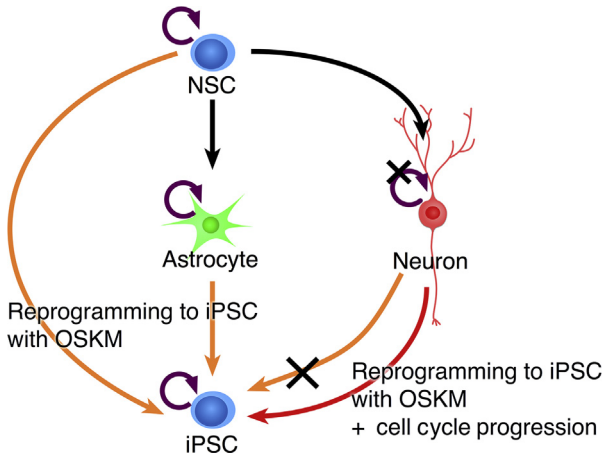

FIGURE 4.4

Reprogramming competency of NSCs and their derivatives. The ratio of successful reprogramming by OSKM varies depending on the cell type. In the neural cell lineage, NSCs and astrocytes could be reprogrammed to iPSCs by OSKM overexpression. OPCs might be reprogrammed to NSCs, but it has not yet been shown whether OPCs can be reprogrammed to iPSCs with OSKM. In contrast, postmitotic neurons show extremely low efficiency of reprogramming to iPSCs with OSKM. Cell-cycle progression together with OSKM enables reprogramming of postmitotic neurons into iPSCs. Arrows indicate the spontaneous, OSKM-induced, and OSKM plus cell-cycle upregulator-induced pathways of cell-fate choice, respectively. The cells with a dark clockwise arrow represent proliferating cells. One of the reasons for the robustness of neuronal identity might be that neurons do not proliferate.

Indeed, the epigenetic status can change dramatically at the point of DNA synthesis during the cell cycle. During the process of DNA replication, the chromatin complex on the original DNA is disassembled and distributed to the two daughter DNAs. Then, the chromatin is reassembled on each of the daughter DNAs, partially or fully erasing the epigenetic memory [93,94]. Likewise, methylated CpGs are passed on to one of the two daughter DNAs, allowing a difference between the original DNA and daughter DNA in terms of DNA methylation status. In this way, the epigenetic memory derived from the original cell type is attenuated through the continuous passaging of iPSCs [95], although the epigenetic modification profiles of the iPSCs retain a fraction of the epigenetic marks of the original cell [96]. It is thus possible that cell-cycle progression can induce global epigenetic changes, including those of genes that underlie the robustness of neurons against reprogramming. On the other hand, overexpression of Nanog in place of cell-cycle accelerators further enhances the pluripotency-related gene expression under the OSKM condition [92]. Actually, histone turnover and histone modification affect epigenetic status independently of the cell cycle [46], suggesting that specific epigenetic changes of the pivotal genes for neuronal identity and the robustness of neurons against reprogramming have similar effects as forced cell-cycle progression during reprogramming. To prove this hypothesis, it would be necessary to identify a specific set of genes that would enable neurons to be reprogrammed among the numerous genes under the influence of cell-cycle-dependent and/or neuronal activity-dependent epigenetic changes.

Recent advances in comprehensive analyses using next-generation sequencing-based methods enable us to genome-widely determine the epigenetic status of cells and tissues of interest. Progress in omics technologies has provided tools useful for this purpose, for example, RNA-seq for transcriptome profiling, ChIP-seq for profiling the location of proteins on the genome, whole-genome bisulfite sequencing for DNA methylome profiling, DNaseI-hypersensitive site sequencing for profiling of nucleosome-free regions, and complementary chromosome conformation capture (3C)-based technologies for profiling of the tertiary structure of chromatin. Guo and colleagues examined the change of the DNA methylome of *in vivo* dentate granule neurons in the adult mouse DG before and after synchronized neuronal activation [97]. At about 0.86% and 0.53% of the 219,991 CpG sites examined by this method (~1% of all CpG sites in the mouse genome), neuronal activation could induce both DNA methylation and demethylation. These CpG sites showing plasticity in response to the neuronal activity are mainly restricted to genomic regions related to memory and learning. Nevertheless, studies focusing on CpG sites refractory to neuronal activity at tissue-dependent and differentially methylated regions (T-DMRs) that are characteristic of neurons will also be important.

For treatment of neuronal diseases, epigenomic profiling for assessment of the neuronal identity from the viewpoint of clinical pathology will also be required. For example, in order to generate genuine neurons suitable for transplantation to patients, it will be indispensable to focus on not only the neuronal properties but also the neuronal epigenomic profiles in detail. Thanks to many studies, the generation of neurons has been achieved, and we can reprogram fibroblasts and iPSCs into several neuronal subtypes with the desired neuronal properties [98]. However, neuronal subtypes residing in different brain regions show physiological and morphological differences from each other even if they share a common neurotransmitter phenotype [99]. Comparison of the cells before and after reprogramming revealed that residual epigenetic memory inherited from the original cells partially remained even after the observable phenotypic change [96]. Therefore, there are potential differences among the endogenous neurons, iPSC-derived neurons, and induced neuronal (iN) cells that have been generated recently using direct reprogramming factors [98]. Reprogrammed cells thus might not behave exactly

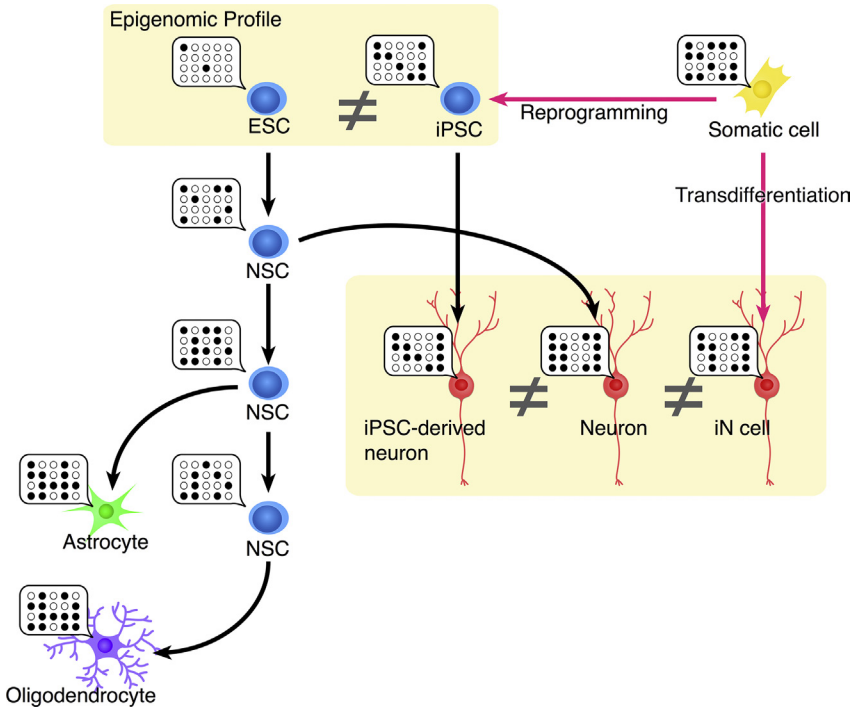

FIGURE 4.5

Epigenomic profiles underlying neural cell identities. Each cell has its own epigenomic profile. During differentiation, this epigenomic profile changes to a different profile depending on the combination of epigenetic modifiers and the initial epigenetic profile. It should be noted that traces of the epigenetic marks of the original cell remain after reprogramming to iPSCs or iN cells from somatic cells by transcription factor-based reprogramming, and after differentiation from iPSs to neurons. It is thus most likely that the epigenomic profiles of endogenous neurons are substantially different from those of reprogrammed neurons.

the same as endogenous neurons *in vivo* (Figure 4.5). Accumulating information about these various kinds of epigenomic profiles in neurons and their functional consequences could lead to a genuine understanding of the roles of epigenetic control in neurons.

Concluding remarks

The capacities of NSCs to differentiate into neurons and glial cells change as cortical development proceeds. Spatiotemporal gain and loss of the differentiation capacities are defined by both a set of epigenetic modifiers and a given epigenomic profile. In this way, a neuron acquires a robust or refractory cell identity, making it difficult to be reprogrammed into other lineages. However, even in such terminally differentiated cells, the epigenomic profile can be rewritten by a set of epigenetic modifiers. Key elements of the sequence-specific alterations of epigenomic status include [1] a combination

of *trans*-acting factors, [2] local chromatin structures, and [3] linkage between *trans*-acting factors and *cis*-regulatory DNA elements. Manipulations of these three factors and cell-cycle progression will lead to the ability to generate a particular neural network and even to "jailbreaking" of the neural cell lineage, which can be applied for medical purposes. Understanding the plasticity and robustness of epigenetic regulations at the molecular level should be further pursued together with elucidating the mechanisms of the brain functions that underlie animal behaviors.

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Glossary

BDNF BDNF is a secreted protein that is involved in synaptic plasticity and memory formation. BDNF encourages the differentiation of neural stem cells into neurons. In postmitotic neurons, BDNF increases synapses and supports their survival. *BDNF* has eight alternative 5′ non-coding exons and a common 3′ coding exon, which are driven by the individual promoters for spatiotemporal expression.

Histone lysine mono-, di-, and trimethylation Histone lysine methylation is catalyzed by a family of proteins that contain a SET domain. Histone lysine mono-, di-, or trimethylation is performed by the addition of one, two, or three methyl groups ($–CH_3$) to the nitrogen of the lysine amine group ($–NH_3^+$), respectively.

iPSC Induced pluripotent stem cells, called iPSCs, are pluripotent stem cells artificially derived from somatic cells by forced expression of specific transcription factors, such as *Oct3/4*, *Sox2*, c-*Myc*, and *Klf4*. Similarly to embryonic stem cells (ESCs), iPSCs have potential to self-renew and develop into any type of cells. The methods for reprogramming to iPSCs from mouse and human cells were established by Yamanaka and colleagues in 2006 and in 2007, respectively. Since this landmark study, many researchers have also established various methods for reprogramming that bypass iPSC generation and direct the production of various cell types by forced expression of a specific set of transcription factors (direct reprogramming).

LTP Long-term potentiation (LTP) is a long-lasting enhancement in signal transmission between two neurons. LTP has two distinct phases: early phase (E-LTP; lasting 1 to approximately 3 hours) and late phase (L-LTP; lasting at least 24 hours). E-LTP is followed by L-LTP. Whereas E-LTP is independent of protein synthesis, L-LTP requires mRNA and protein synthesis in the postsynaptic cell. LTP is induced by high-frequency stimulation of the neuron leading to elevation of Ca^{2+} in postsynaptic dendritic spines and a prolonged enhancement in signal transmission across the synaptic cleft. In this process, protein kinases, such as CaMK-II and ERK, are activated, followed by changes in gene expression and protein synthesis.

Myelination Myelination is one of the characteristics observed during oligodendrocyte maturation. In culture, oligodendrocytes develop from oligodendrocyte precursors (OPCs) by extending processes and forming sheet-like myelin protein-containing protrusions. In the presence of neuronal axons, OPCs extend their processes, and upon contact with the axon initiate a wrapping process, forming the compact myelin sheath.

Progenitor cell Progenitor cells are any dividing cells with the capacity to differentiate. In contrast to stem cells, progenitor cells include putative stem cells in which self-renewal has not yet been demonstrated.

Stem cell Stem cells are defined as cells with the ability to self-renew and to give rise to daughter cells that have more specialized properties with limited differentiation capacities.

Synaptic plasticity Synaptic plasticity is the ability of synapses to change the structure of the synaptic connections. In response to various signals, the number of receptors located on a synapse increases or decreases. Long-term depression (LTD) and long-term potentiation (LTP) are two of the most studied forms of synaptic plasticity.

CHAPTER

Role of Epigenetics in the Brain

5

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KEY CONCEPTS

- Studies in a range of organisms have linked epigenetic mechanisms to processes associated with brain development and function.
- Studies continue to link epigenetics, cognitive dysfunction, and several psychiatric disorders.
- Compelling evidence also indicates a role of epigenetics in the transgenerational perpetuation of phenotypes.

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ABBREVIATIONS

AD Alzheimer's disease
Arc Activity-regulated cytoskeleton-associated protein
BDNF Brain-derived neurotrophic factor
CpG Cytosine–phosphate–guanine
CR Caloric restriction
CRF Corticotropin-release factor
DG Dentate gyrus
E33 Embryonic day 33
FGF Fibroblast growth factor
fMRI Functional magnetic resonance imaging
GA Gestational age
GAD67 Glutamate decarboxylase 67
Gadd45b Growth arrest and DNA-damage-inducible protein 45 beta
GFAP Glial fibrillary acidic protein
HDACs Histone deacetylases
HPA Hypothalamic–pituitary–adrenal
IGF2 Insulin-like growth factor 2
LGA Large for gestational age
MBD Methyl-CpG-binding domain
MeCP Methyl-CpG-binding protein
miRNAs microRNAs
Nr3c1 Nuclear receptor subfamily 3, group C, member 1
NRSF Neuron-restrictive silencer factor
NSCs Neural stem cells
NTDs Neural tube defects
PTMs Posttranslational modifications
PTSD Posttraumatic stress disorder
RE1 Repressor element 1
REST Repressor element 1 silencing transcription factor
SAH *S*-adenosylhomocysteine
SAM *S*-adenosylmethionine
SGA Small for gestational age
STAT3 Signal transducer and activator of transcription 3
TSST Trier Social Stress Test
VPA Valproic acid

Introduction

Proper brain development and function rely on the coordination of an extraordinarily complex set of neurodevelopmental events that involve genetic and environmental interactions at many biological levels. As brain and behavior development are processes that continue throughout the lifespan through senescence, this interplay also changes and evolves over time. Since the birth of behavioral epigenetics research in the last decade, epigenetics has emerged as a leading candidate for biological pathway linking gene–environment interactions in neurobehavioral development. Advances in molecular biology have certainly given new insights into epigenetic factors that can alter the activity of genes without modifying the underlying sequence of DNA, as well as evidence that these factors play a critical role in allowing the emergence of different phenotypes (at all levels, ranging from molecular to organismal). These factors, too, have emerged as a valuable measure informative of norms and aberrations present at the molecular level, and aberrant epigenetic marking of the genome is now recognized as a leading biological basis for many stress-related disorders and cognitive impairment.

To highlight the literature concerning these themes, we first describe a role for epigenetics in the transgenerational perpetuation of phenotypes and the expression of imprinted genes to highlight that epigenetics has played a critical role in shaping brain evolution and behavior. Second, we integrate animal and clinical research to describe a role for epigenetic mechanisms in specific processes integral to brain development, including neural cell differentiation and neurogenesis. Finally, we end by highlighting the critical role that epigenetics plays in brain function by discussing some of our collaborative work and that from others linking epigenetics to learning and memory, the susceptibility to stress-related disorders, and cognitive impairment.

Role of epigenetics in evolution

From an evolutionary perspective, any mechanism that permits the potential for different phenotypes to be expressed from a single genotype may be of important adaptive significance. Additional implications for evolutionary fitness arise when phenotypic plasticity may be mediated by experience and/or environmental interactions. Dynamic epigenetic changes provide such a mechanism, permitting environmental factors to influence gene expression. From plants to yeasts to humans [1–4], evidence continues to accumulate that epigenetic modifications mediate phenotypic plasticity across the lifespan and can even do so between generations. For example, in humans many epigenetic changes are now known to occur across the lifespan. During the early years of life, monozygotic twins are epigenetically indistinguishable, but as they age, significant differences in levels and distribution of DNA methylation and histone acetylation are known to develop [5]. It is through epigenetic processes that these genetically identical twins can exhibit differential patterns in gene expression, potentially resulting in phenotypic discordance commonly observed in identical twins.

The adaptive significance of epigenetic mechanisms is not limited to altering phenotypes of individuals in response to environmental factors (a topic we visit in more detail in a later section in this chapter). Acquired epigenetic patterns may also be passed on to subsequent generations. The intrauterine environment has long been recognized for its major contributory role in offspring development, and environmental events within this context are known for their canalizing effects on behavioral development. For example, exposure of pregnant women to war-related stressors (including dietary restrictions) in the 1940s has been associated with an increase in prevalence of schizophrenia and anxiety disorders in their children [6]. Behavioral epigenetic studies focusing on the developmental origin of health and disease hypothesis have begun to identify links among early nutrition, epigenetic processes, and phenotypic outcomes, and here we highlight two lines of this work. First, individuals who were prenatally exposed to famine during the Dutch Hunger Winter in 1944–45 have less methylation of DNA associated with an important imprinted gene regulating body growth (insulin-like growth factor 2, IGF2) in comparison to unexposed, same-sex siblings, an effect present six decades from the initial exposure [7]. Second, a study utilizing a Gambian cohort where diet varies greatly according to season has shown that DNA methylation patterns in first-generation offspring differ depending on season of conception [8].

Some of the first experimental evidence for epigenetically dependent transgenerational phenotypic expression was observed in the agouti mouse [9]. In these classic studies, a diet rich in methyl donors (methionine, folate) influenced methylation status in the mother which resulted in different coat color and feeding behavior in the offspring. Similarly, in our own studies on early-life adversity in rats, we have shown that repeatedly exposing one-week-old infants to a stressed caretaker with limited bedding material is sufficient to alter epigenetic regulation of the brain-derived neurotrophic factor (BDNF) gene, not just within the generation directly exposed to our manipulations [10,11] but also in the next generation [11]. For example, female infants subjected to our caregiver manipulations displayed significant methylation of BDNF DNA within their prefrontal cortex as adults (Figure 5.1A), and even showed the same types of aversive caregiving behaviors toward their own offspring that they had experienced themselves as infants. In their eight-day-old offspring (both males and females) we also found significant methylation of BDNF DNA in their prefrontal cortex (Figure 5.1b). Surprisingly, cross-fostering experiments (where offspring born to maltreated-females were cross-fostered to normal females and *vice versa*) indicated that the presence of these methylation marks was

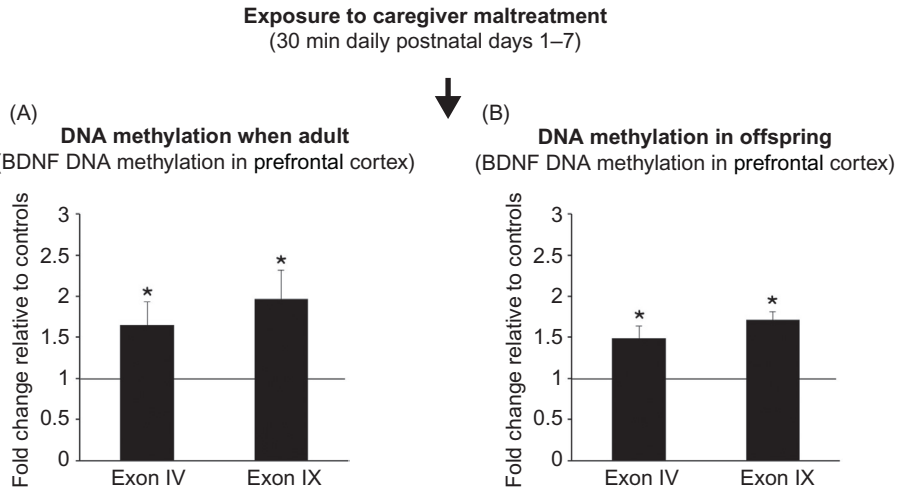

FIGURE 5.1

Epigenetic effects of early-life adversity in two generations of rats. (A) In the 2009 Roth et al. study [11], we showed that brief but repeated bouts of caregiver maltreatment during the first 7 days of life influenced adult levels of BDNF DNA methylation (increased) in the prefrontal cortex. Control groups (represented by the solid line at 1) included rats exposed to nurturing care outside of the home cage or only nurturing care from the biological mother. (B) We also took maltreated females and bred them to produce a second generation of infants. Females were observed to mistreat infants within their home cage, and both their male and female offspring likewise showed altered DNA methylation (increased on postnatal day 8).

not exclusively attributable to the postnatal caregiving environment. Finally, another line of elegant work demonstrating transgenerational epigenetic consequences has focused on the negative impact of the environmental toxin vinclozolin on offspring development [12]. It has been shown that vinclozolin exposure during early development influences epigenetic programming of the germ line, influencing gene expression in the amygdala and resulting in changes in anxiety-related behaviors in offspring as far as four generations beyond the initial toxin exposure.

Evolutionary implications for epigenetics also come from the fact that epigenetic modifications are also heritable in the form of imprinted genes. Imprinted genes are expressed in an origin-dependent manner such that imprinted alleles are epigenetically silenced (via DNA methylation), limiting expression to the non-imprinted alleles from either the mother or the father. Imprinting control regions are dominated by the matriline, and these regions can determine both maternal and paternal gene expression patterns [13,14]. From an evolutionary standpoint, the greater imprint control of the mother would appear adaptive, especially for mammals, given the levels of female parental investment and influence on offspring development, both prenatally and postnatally [15].

The evolution of the mammalian brain has long been a topic of interest, but more recently studies exploring the potential role of epigenetic molecular mechanisms in this process are receiving deserved attention [16,17]. One of the more observable trends in mammalian brain evolution is the increase of encephalization, especially among primates. Although specific epigenetic roles in this

phenomenon remain unclear, a strong maternal influence is apparent. Indeed, it has been hypothesized that the most important selection factor in primate brain evolution is related to the extensive postnatal neocortical development during a time period of close relationships with the mother [18]. It is also interesting that imprinted genes are not expressed in monotremes or birds, perhaps suggesting that viviparity was an important factor shaping genomic imprinting in placental mammals [19]. As elegantly discussed by Keverne [16,17], viviparity would uniquely allow selection pressures to operate over three generations (mother, developing offspring, and developing oocytes) of coexisting matrilineal genomes, with many imprinted genes co-expressed in the placenta and hypothalamus. These genes could then play a role in regulating the co-adaptive functions of both structures, as the placental hormones and hypothalamus both strongly influence maternal care and mother–offspring interactions.

Role of epigenetics in brain development

Development of the human brain is a long-lasting process, beginning with the emergence and differentiation of neural structures and their functions [20]. Complex sets of gene–environment interactions are essential for these early processes and normal functioning throughout the lifespan, and disruptions at any of these developmental points, especially early on, can have profound consequences on behavioral outcomes. Research regarding the precise timing of ontogenetic events during human brain development is highly valuable because it will enable us to understand, for example, mechanisms involved in the onset of psychiatric disorders that emerge from disruptions in normal neural functioning [21]. As we will see later in this chapter, recent research has revealed that epigenetic mechanisms are heavily involved in regulating stress-related outcomes.

Brain development

To appreciate the role of epigenetic mechanisms in processes underlying normal and abnormal brain development, it is first necessary to describe the processes involved in brain development. Brain development begins around gestational age (GA) weeks 2 to 3 with the formation of the neural tube [20,22]. During this process, neural stem cells (NSCs) differentiate and orient themselves along part of the ectoderm called the neural plate. The neural groove then forms in the neural plate, and the walls of the groove gradually move together and fuse, first in the center of the neural tube and then continuing in both the rostral and caudal directions. As the neural folds come together, part of the ectoderm is pinched off and the neural crest is formed. This is where all neurons with cell bodies in the peripheral nervous system are derived. When the neural tube is fused completely, a single layer of cells forms from the NSCs, known as the ventricular zone. As development progresses, this region eventually forms the ventricular system of the brain. The NSCs in the most rostral region of the neural tube will give rise to the brain, while more caudally positioned cells will give rise to the hindbrain and spinal column. Throughout the next month after formation of the neural tube, the embryo undergoes rapid growth, increasing tenfold by the end of GA week 8 [20]. During this time, the three primary brain vesicles begin to form as well. These segments further subdivide, and by the end of the embryonic period the five secondary brain vesicles are present, thus establishing the primary organization of the central nervous system [23].

Neural stem cells have the ability to self-renew and generate into the three major cell types in the central nervous system: neurons, astrocytes, and oligodendrocytes [24,25]. NSCs divide symmetrically in early gestation to increase their own numbers, and then later undergo neurogenesis by asymmetric cell division. Neurogenesis begins around embryonic day 33 (E33) in humans and E10 in rodents [26]. This type of cell division gives rise to two distinct daughter cells: another NSC and a neuron. The new NSC remains in the proliferative zone and continues to divide, while the neuron takes its place in the developing brain. Later, NSCs eventually gain multipotentiality and acquire the ability to generate astrocytes and myelin-producing oligodendrocytes as well as neurons. Until recently, it was thought that there was much greater precursor diversity in primates than in lower vertebrates. Evidence has now shown that the ventricular zone of rodents also contains a variety of precursor cells, which become progressively restricted in their differentiation potential as development proceeds [26].

One of the structures that best illustrates migration during brain development is the cerebral cortex. The migration of neurons into the developing cortex results in the formation of its six-layered structure, which serves as a basis for numerous higher-order perceptual and cognitive functions [20,27]. The first neurons to leave the proliferative zone initially form a primitive structure called the preplate. Preplate cells form temporary connections that serve as placeholders until the appropriate neurons are in place and ready to form more mature connections [28]. Once the preplate is complete, it splits into two separate regions—the marginal zone and the subplate—and then from these regions the cortical plate emerges. The first neurons to arrive in the cortical plate will form cortical layer VI, the deepest layer of cortex, and later migrating cells will form more superficial layers of cortex, creating an inside-out order of migration. The cortical plate will produce cortical layers II to VI, whereas the marginal zone will ultimately form cortical layer I. At GA week 32, the developing cortex has a full adult complement of distinct vertical lamina, containing afferents of all the major neurotransmitter systems and a diversity of differentiated glia and neuronal cell types [22].

Once positioned in the cortex, neurons begin to extend their dendritic and axonal processes that form fiber pathways of neural networks. The efficiency of information transmission in the pathways is greatly enhanced by myelin, which layers the axons. Between GA weeks 20 and 28, mature myelin is detected first in subcortical regions and later in cortical regions [20]. Neurotrophic factors and growth factors such as BDNF influence the migration or retraction of neurons [29]. It has been found in rodents that the expression of neurotrophic factors reaches the highest levels during the prenatal period as neurons first establish synaptic connections but continue to be expressed in a regionally specific manner during postnatal development. They play a pivotal role in synaptic plasticity throughout the lifespan and are vital for neuronal changes associated with, for example, memory formation and responsivity to drugs of abuse.

The number of neurons in the human brain reaches its maximum at GA week 28, and this overproduction phase is hypothesized to maximize the capacity of the immature brain to store information [22]. However, elimination of these neurons later in life is a mechanism by which the brain regulates the establishment of effective and functional neural circuits. As many as half of the neurons produced during neurogenesis die in the process of apoptosis, or naturally occurring cell death, by the end of adolescence. Most naturally occurring cell death in neuronal populations occurs prenatally, while both cell death in glia populations and the events involving exuberant production and pruning of connections largely occur in the postnatal period.

Early postnatal life represents a time of marked change in brain structure and function. The human brain grows to about 70% of its adult size by age 1 and about 80% of adult size by age 2, and it reaches approximately 90% of adult volume by age 6 [30,31]. The exuberant connectivity that is a hallmark of early development is gradually pruned back through competitive processes that are influenced by the experience of the organism. Together with inborn genetic factors, these early experience-dependent processes act on an immature brain to trigger further development of neural circuits. Over time, experience plays an increasingly more prominent role in the shaping of neural circuitry.

In the postnatal period, neurogenesis continues to a limited degree. Adult NSCs have been found in the two principal neurogenic regions: the subgranular zone of the hippocampus and the subventricular zone [20]. These unique forms of neurogenesis appear to continue throughout adult life but produce only a small proportion of the neuronal population. In contrast, proliferation and migration of glial progenitors, although beginning prenatally, are largely postnatal processes and continue for an extended period as oligodendrocytes and astrocytes differentiate. Unlike neurons, it appears that they can persist indefinitely in the adult brain in a wide anatomical distribution.

Brain imaging is providing important clues about the time course of age-related biological alterations in the brain and provides an opportunity to link these changes to evolving behavior. For example, correlational analyses of functional magnetic resonance imaging (fMRI) data acquired at rest have recently been used to study developmental changes in functional connectivity in older children and adolescents. These studies suggest that there are two types of age-related changes in functional connectivity: (1) decreases in local connectivity among anatomically adjacent but functionally distinct brain regions as they are integrated into their respective networks, and (2) increases in long-range connectivity among networks [22]. Additionally, by comparing volumes of brain structures across the lifespan, it has been found that during childhood and adolescence changes in brain structure are at least as striking as those at the end of life [32]. These studies found age-related decreases in the volume of the frontal cortex, thalamus, and nucleus accumbens across the lifespan, as well as increases in cerebral white matter volume during childhood and early adulthood that give way to decreases later in life. Unfortunately, much uncertainty remains regarding the timing of proliferation, migration, differentiation, and regression during the postnatal period in humans and the timing of these processes in relation to each other. Although we have gained insight on postnatal development in humans, the data consist mainly of postmortem and neuroimaging studies, which are both limited and inherently constrained. However, the remarkable evolutionary conservation of neurodevelopmental events and their timing across species such as rodents help to validate the extension of knowledge about brain development from animals to human beings [26].

Epigenetics in early brain development

As should be evident from the aforementioned discussion, the structure and function of neural circuits continually change and evolve as brain development progresses, responding to intrinsic genetic programs and extracellular cues from the environment. Of recent interest in the neuroscience field is the role of epigenetic mechanisms, which include DNA methylation, histone modifications, and non-coding RNA-mediated processes in brain development and function (the details of these epigenetic processes are not reviewed here in detail but can be found in other sections of this book). Understanding typical trajectories for the maturation of healthy neural circuits and how the interplay of genes

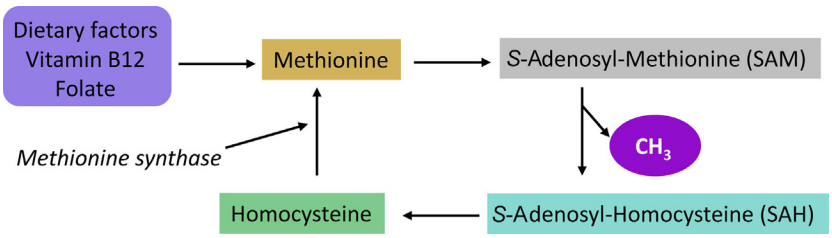

FIGURE 5.2

Schematic of the methylation cycle. *S*-Adenosylmethionine (SAM) is generated from methionine. Conversion of SAM to *S*-adenosylhomocysteine (SAH) produces methyl groups then available for DNA methylation. Finally, SAH can be hydrolyzed to form homocysteine, which in turn can be remethylated. This cycle is all under the influence of folate and vitamin B12.

and environment affect these circuits is critical in identifying developmental origins of mental and somatic illnesses.

As previously mentioned, neural tube formation occurs very early in the prenatal period, and failure in initial fusion or continuation of closure results in neural tube defects (NTDs). Research indicates that dysregulation of gene expression through epigenetic mechanisms could increase the risk of human NTDs [33]. Evidence suggests a link between an impaired methylation cycle (see Figure 5.2) and human NTDs, including the association with higher levels of the one-carbon metabolite, homocysteine, and lower levels of folate and/or vitamin B12 in maternal blood. Additionally, inhibition of choline uptake or metabolism causes NTDs in cultured mouse embryos, and choline deficiency is known to alter DNA and histone methylation patterns in fetal brain tissue [34,35]. It was also found that exposure to methylation cycle inhibitors or excess methionine causes cranial NTDs, and both of these treatments result in a reduced ratio of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH), which signifies reduced methylation potential [36]. Taken together, it seems that a number of factors could epigenetically modify development of the neural tube and influence susceptibility to NTDs.

The majority of current epigenetic research related to brain development has focused on the differentiation and fate specification of NSCs. It seems that DNA methylation in astrocyte-specific gene promoters is a critical factor in enabling NSCs to differentiate into astrocytes during development. For example, it has been found that astrocyte differentiation is largely dependent on the activation of a transcription factor: signal transducer and activator of transcription 3 (STAT3). One particular study compared the methylation status of the binding site of STAT3 in the promoter of glial fibrillary acidic protein (GFAP), which is highly expressed in astrocytes, between mid (E11.5) and late (E14.5) stages of development in mice [37]. It was revealed that the STAT3 binding site was hypermethylated in E11.5 neuroepithelial cells, but hypomethylated in E14.5 cells and responsive to the STAT3 activation signal needed to express GFAP. Overall, methylation led to inaccessibility of STAT3 to the binding element, which makes NSCs unable to differentiate into astrocytes earlier in gestation. Furthermore, the genome-wide DNA methylation status of E11.5 and E14.5 NSCs has been compared, confirming that many astrocytic genes become demethylated [38].

Methyl-CpG-binding domain (MBD) proteins, critical for CNS development and function and highly expressed in neurons, are also involved in astrocytic differentiation, specifically the silencing of astrocytic genes in neurons to restrict the cell fate. For example, aberrant expression of MBDs inhibits astrocytic gene expression and differentiation of embryonic neuroepithelial cells, which normally differentiate into astrocytes under the control of STAT3 activation [39]. The exon 1 region of the GFAP gene remains highly methylated, even in late-stage neuroepithelial cells that have already lost methylation in the STAT3 binding site within the GFAP promoter. MeCP2, an MBD protein that binds to DNA that has been methylated, binds to the highly methylated exon 1 of the GFAP gene in neurons, as well as hypermethylated CG sites elsewhere.

Histone modifications are another epigenetic mechanism that influence NSC differentiation and fate. Histone deacetylases (HDACs) are a class of enzymes that condense chromatin, thus resulting in transcriptional repression [40]. The antiepileptic valproic acid (VPA) functions as an inhibitor of HDACs, and VPA has been shown to induce neuronal differentiation of adult NSCs [41]. In addition, VPA can inhibit glial cell differentiation of NSCs, even under conditions that favor lineage-specific differentiation. This occurs by upregulation of neuron-specific genes, such as the neurogenic basic helix–loop–helix transcription factor NeuroD. It was found that overexpression of NeuroD in NSCs induced neuronal differentiation and suppressed glial differentiation. Overall, this research suggests that HDAC inhibitors such as VPA promote and inhibit neuronal and glial fate, respectively, through induction of transcription factors.

Oligodendrocytic differentiation is distinct from neural or astrocytic differentiation because it involves global histone deacetylation to be initiated [42]. A recent report identified the types of HDACs involved in oligodendrocyte differentiation [43]. HDAC1:HDAC2 double mutant mice, but not HDAC1 or HDAC2 single knockouts, developed severe defects in oligodendrocyte differentiation, without the neuronal or astrocytic cell population being affected. It is thought that HDAC1 and 2 regulate oligodendrocyte differentiation by the Wnt-signaling pathway via limiting the generation of oligodendrocytes from NSCs, as decreasing Wnt signaling in NSCs results in early production of oligodendrocytes [44]. In support of this global histone deacetylation, it is not surprising that the treatment of NSCs with HDAC inhibitors led to a reduced amount of oligodendrocytes and increased numbers of neurons and astrocytes.

Noncoding RNAs are increasingly recognized as exerting epigenetic effects on gene regulation, and in particular it has been demonstrated that microRNAs (miRNAs) play a role in NSC differentiation and fate specification. miR-124a is expressed predominantly in neural tissues and has been shown to participate in the differentiation of NSCs into neurons by mediating degradation of non-neuronal gene transcripts [45]. The expression of miR-124a is regulated by the repressor element 1 (RE1) silencing transcription factor (REST)/neuron-restrictive silencer factor (NRSF) protein complex, which is expressed only in NSCs and some non-neuronal cells. In NSCs, NRSF/REST suppresses expression of the miR-124a gene, which allows the stability of non-neuronal gene transcripts to be increased, thus preventing NSCs from differentiating into neurons. When NRSF/REST is absent, the expression of miR-124a and neuronal genes is upregulated, leading to a preference for neuronal fate. Furthermore, it has been shown that overexpression of both miR-124 and miR-9, another neural tissue-specific miRNA that can downregulate NRSF/REST, promotes neuronal differentiation [45]. Other miRNAs, such as miR-128, miR-129, and miR-298, are also expressed exclusively in the neuronal lineage, while miR-23 is solely expressed in the glial lineage, and miR-26 and

miR-29 are more strongly expressed in glia than in neurons [46]. This research indicates that altered expression of individual miRNAs may be cellular context dependent and critical for cell-fate choice [24,40]. Altogether, epigenetic alterations appear to serve as coordinating systems for regulating gene expression during each stage of neural cell differentiation [40]. In the future, this new knowledge may allow us to reprogram or modify the fate of stem cells, using specific treatments at certain time points to change their epigenetic profiles.

Finally, a largely unexplored but promising area of research for the involvement of epigenetic regulation is the sequential development of cortical layers [27]. Specifically, histone methylation has been shown to alter the production ratio of deep layer and upper layer neurons [47]. Histone acetylation is also potentially important during cortical development. VPA induced neuronal differentiation and selectively augmented the neuronal population of upper layers in mouse embryonic stem cell-derived NSCs [48]. It has been proposed that HDAC inhibition promotes the progression of neuronal differentiation during cortical development, switching deep-layer-producing NSCs into upper-layer-producing NSCs.

Epigenetics in adult neurogenesis and later brain maturation

Just as epigenetic mechanisms are involved in the differentiation of NSCs early in development, they are also involved in adult neurogenesis [49]. One of the first studies to recognize this was from mice lacking methyl-CpG-binding domain protein 1 (MBD1), which is normally expressed throughout the adult brain, with the highest concentration in the hippocampus. These mice develop normally and appear healthy throughout life, but have decreased neurogenesis and increased chromosomal instability in the dentate gyrus [50]. It was later shown that MBD1 facilitates neuronal differentiation by direct binding to the promoter basic fibroblast growth factor 2 (FGF2). MBD1-induced methylation of the FGF2 promoter results in downregulation of FGF2 expression, thus allowing hippocampal NSCs to differentiate into neurons [51]. Another study looked at the role of growth arrest and DNA-damage-inducible protein 45 beta (Gadd45b), which was identified as a neural activity-induced immediate early gene in mature hippocampal neurons [25]. It was found that Gadd45b knockout mice displayed deficits in neural activity-induced proliferation of neural progenitors and dendritic growth of new neurons in the adult hippocampus. It was also concluded that Gadd45b activity induces neurogenesis in part by DNA demethylation in the promoter regions of *BDNF* and *FGF1*, two genes that are important for neurogenesis. Histone modifications also seem to be important for neurogenesis, and the existence of both activating and repressive histone methylation marks is crucial for the rapid activation of genes during differentiation [52].

A considerable amount of research has been dedicated to understanding early brain development and more recently adult neurogenesis, but much less is known about how development continues into aging and senescence. What is clear, though, is that DNA methylation patterns change across the lifespan, with significant brain changes occurring in aging, including large-scale DNA hypomethylation and, in some specific structures, gene promoter-specific DNA hypermethylation [42,53]. For example, Siegmund et al. [54] observed in the cerebral cortex a progressive rise in DNA methylation levels across the lifespan for a number of genes related to CNS growth and development, and this corresponded with declining levels of their mRNAs. Further, other genes were defined by a sharp rise in DNA methylation levels within the first few months or years after birth. Hernandez et al. [55] found a positive correlation between age and DNA methylation in the

frontal cortex, temporal cortex, pons, and cerebellum. They also found that the CG sites where the DNA methylation level is significantly associated with age are physically close to genes involved in DNA binding and regulation of transcription. Finally, the results of a new study confirm reconfiguration of DNA methylation patterns throughout the lifespan and show that DNA methylation is highly dynamic in frontal cortex brain cells of both mice and humans during the transition from birth to adulthood [56]. For example, from fetal to young adulthood, a time in development when synapses are growing rapidly, investigators found that non-CG methylation accumulates in neurons (but not glia).

Epigenetics and extrinsic factors regulating brain development

Researchers are also learning other ways in which experiences and epigenetic factors work together to alter aspects of brain development. It has been found that age-related effects on projection neuron dendrites can be reversed in rodents through environmental enrichment [57]. Specifically, the age-related loss of dendritic spines on projection neurons, which is a factor that inhibits proper neuron function, was improved in aging rats exposed to an enriched housing environment. It has also been found that age-related changes in DNMT3a, which catalyzes DNA methylation, were diminished in the aging mouse hippocampus for mice with a diet restricted in calories [58]. Caloric restriction (CR) has been suggested as a strategy to decrease age-related alterations in the brain, and the findings from this study imply that these changes may have a significant impact on gene expression and associated cognitive functioning. CR lowers stress levels leading to adaptive responses, such as enhanced defense mechanisms and inflammatory responses, and delays the onset of age-related diseases such as cardiovascular diseases, type 2 diabetes, and cancer [59].

Gestation has long been recognized as a critical period for programming lifelong alterations in offspring physiology. Diverse prenatal exposures such as maternal diet, stress, infection, and drug use may modulate epigenetic mechanisms which in turn could mediate developmental programming of adult disease and aging [59]. After the prenatal period, the size of an infant at birth, a measure of gestational growth, has long been recognized as a biomarker of future risk of morbidity. Both being born small for gestational age (SGA) and being born large for gestational age (LGA) are associated with increased rates of obesity and metabolic disorder, as well as a number of mental disorders. Overall, research demonstrates that aberrant epigenetic mechanisms in the brains of SGA and LGA offspring lead to cellular disruptions in development and altered gene expression in adulthood [60]. Animal models of SGA and LGA have identified deficits in learning and memory, and these deficits are correlated with altered BDNF signaling in the brain and placenta in both SGA and LGA pregnancies [61]. BDNF signaling is a crucial mechanism for altering DNA methylation in neurons, and this mechanism is heavily involved in controlling hippocampal learning and memory as well as hypothalamic function. Additionally, both LGA and SGA are associated with similar patterns of global DNA hypomethylation across multiple brain regions [62]. These findings are troubling considering that significant adverse neurological outcomes have been associated with global hypomethylation, including neurodegeneration, altered neuronal gene expression, and severe behavioral deficits [60]. Altogether, understanding the underlying molecular mechanisms of aging and the ways in which early-life exposures influence these mechanisms will serve as a basis for development of clinical therapies or nutrition/lifestyle interventions to alleviate the adverse consequences of early-life programming and contribute to healthier aging.

Role of epigenetic mechanisms in brain function

Biologists studying developmental processes associated with embryonic development and cancer have long recognized the importance of epigenetic marks in producing stable changes in gene activity that, for example, underlie whether a stem cell differentiates into a neuron or the growth of tumors in the body. Work with the marine mollusk *Aplysiacali fornica* provided some of the first evidence that epigenetics (particularly histone acetylation) plays a role in synaptic plasticity [63]. It was not, however, until after work from late in the last decade emerged that epigenetic mechanisms, particularly DNA methylation, began receiving considerable attention as a framework to understand how environmental factors can interact with genes to promote changes in neural function and cognition. Studies since have also sought to determine whether aberrant epigenetic marking of the genome is a biological basis for stress-related disorders and cognitive impairment (which are topics that will be visited in much more detail in subsequent chapters within this book).

Epigenetics and memory

One of the main interpretations of epigenetic mechanisms with regard to brain function relevant to learning and memory is that environmentally driven epigenetic tags are able to alter gene expression, creating functional changes in neurons and circuits that can facilitate memory formation and thus long-term behavioral change. With regard to DNA methylation, we draw on the pioneering work of David Sweatt and his colleagues, who first used hippocampal brain slices in combination with DNA methylation inhibitors and then used fear-conditioning paradigms and adult rats. Using hippocampal brain slices, their earliest work showed that inhibitors of DNA methylation in adult CNS tissue could alter methylation patterns of plasticity genes (*BDNF* and reelin) and block hippocampal long-term potentiation [64].

In subsequent work in fear-conditioned animals, Sweatt and colleagues showed a host of rapid changes in methylation states that included exon-specific demethylation of BDNF DNA in area CA1 of the hippocampus [65]. They later demonstrated that changes in DNA methylation involved in memory formation were much longer lived and not exclusive to the hippocampus, showing, for example, hypermethylation of a memory suppressor (calcineurin gene) within the dorsomedial prefrontal cortex, an effect remarkably present 30 days after conditioning [66]. Finally, helping to establish a necessary role between DNA methylation changes and fear memory formation, they demonstrated that pharmacological manipulation of DNA methylation (via use of DNMT inhibitors) disrupted fear memory formation. This relationship was later replicated in conditional DNMT1 and DNMT3a knockout animals in another laboratory [67]. Additional work in rodents has corroborated the role of DNA methylation in neural processes, supporting other forms of learning and memory, including novel object recognition [68], successful navigation of the Morris water maze [69], and associative reward learning [70]. Histone modifications are another mechanism that has been explored for their role in learning and memory, and several lines of work indicate that global and gene-specific increases in histone acetylation and phosphorylation are essential components of synaptic plasticity and memory formation [71,72].

Epigenetics and stress

A second main interpretation of epigenetic mechanisms with regard to the brain is that epigenetic changes provide a route through which traumatic experiences can moderate the effects of genes and render an individual susceptible to stress-related disorders. Early evidence in support of this notion came from studies looking at methylation in the hippocampus of males who had committed suicide and had a history of child abuse; in these studies, investigators found increased methylation of Nr3c1 and ribosomal RNA genes [73,74]. Work since has shown that males suffering severe childhood abuse also possess genome-wide changes in hippocampal DNA methylation, including hypermethylation of over 200 gene promoters and hypomethylation of around 100 gene promoters, many of which are involved in neural plasticity [75]. We have subsequently learned that these modifications (albeit, detected peripherally) are also present in children subjected to institutional care [76], adults who experienced loss of a parent or maltreatment during their childhood years [77], and adults who experienced a disadvantaged socioeconomic position during childhood [78]. Additional support for a role of epigenetics in stress-related disorders that emerged relatively early in the literature came from postmortem work involving individuals who had committed suicide and/or had been diagnosed with major depression. For example, these individuals were found to have significant alterations in levels of DNMT mRNA [79] and increased methylation of BDNF DNA [80]. Subsequent work has shown altered DNA methylation of numerous genes that play a role in neuronal growth, development, and cholinergic transmission [81]. Studies have also shown strong correlations among child abuse/life stressors, methylation states of immune- and synaptic plasticity-related genes, and the diagnosis of posttraumatic stress disorder (PTSD) [82,83]. Finally, the picture is beginning to emerge that epigenetic marks in maltreated individuals can be changed through psychotherapeutic approaches, with consequences for cognitive function [84].

The aforementioned studies have looked at resultant epigenetic aberrations a considerable length of time after a significant traumatic experience or series of stressful events, but recent experimental work in laboratory settings using the Trier Social Stress Test (TSST) have begun to illustrate the ability of mildly stressful experiences to evoke rapid epigenetic changes in the human genome. In one such study, investigators found in blood samples from participants following the TSST a short-lived increase in methylation of the oxytocin receptor gene, but this modification was no longer present 90 minutes poststress [85]. Consistent with the fact that the response to the TSST is known to differ for males and females, another study has reported greater methylation of the *Nr3c1* gene after the TSST in females compared with males, which coincided with a decrease in salivary cortisol released during the TSST [86]. Other reports helping to experimentally establish a link between epigenetic patterns and brain function include one demonstrating that greater stress and lower methylation of catechol-*O*-methyltransferase Val^{158} allele are correlated with more inefficient prefrontal activity [87], and a second showing that DNA methylation of the gene encoding the oxytocin receptor is associated with individual variability in neural responses within brain regions supporting social perception [88].

This second interpretation of the role of epigenetics in brain function is also empirically supported by studies on rodents. A growing body of work has revealed that alterations in CNS DNA methylation serve as markers of not only memory but also exposure to chronic stress. For example, chronic social defeat in mice has been commonly used to study the genetic precursors of stress-related psychiatric

disorders, particularly depression, but has recently been recognized for its utility in elucidating epigenetic precursors. In the social defeat paradigm, mice are subjected to repeated aggressive encounters with a conspecific, which results in increased avoidance of social contact. Susceptible mice, or mice that spend less time in a social interaction zone after social defeat, have been found to display long-lived demethylation of the corticotropin-release factor (CRF) gene in the paraventricular nucleus, which produces an overactive hypothalamic–pituitary–adrenal (HPA) axis and social avoidance behaviors [89]. Resilient mice, on the other hand, spend more time in the social interaction zone after defeat and do not display the same epigenetic changes.

We recently explored this phenomenon using a stress regimen known to produce physiological and behavioral outcomes in adult rodents that are remarkably similar to those seen in PTSD patients, including increased cardiovascular and corticosteroid reactivity, increased anxiety, exaggerated startle, and lasting cognitive impairments [90]. To provide a sense of lack of control and a traumatic experience, rats were immobilized while they were inescapably exposed to a cat, a predatory stimulus that perturbs hippocampal and amygdaloid activity and evokes immense fear-like behavior. To model re-experiencing of the threatening situation, rats were given a second inescapable cat exposure episode 10 days after the first. Finally, to model a lack of social support, a new contributor to PTSD, housing conditions of rats were continually disturbed and randomized over the entire psychosocial

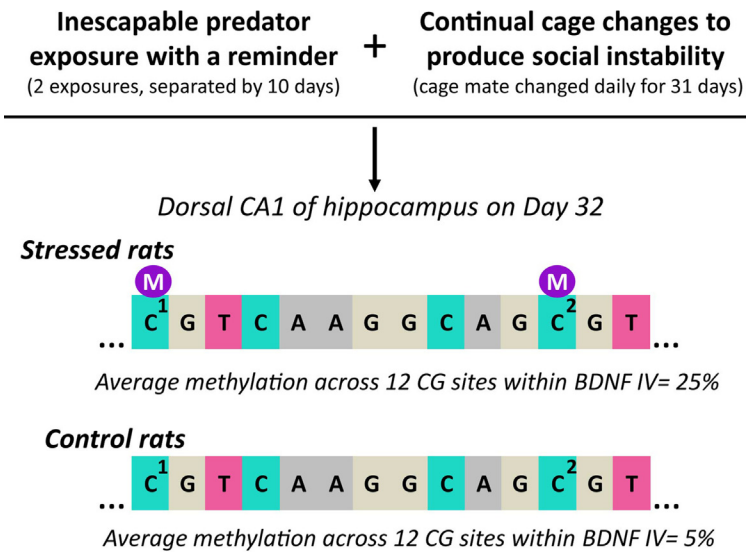
Inescapable predator exposure with a reminder
(2 exposures, separated by 10 days)
+
Continual cage changes to produce social instability
(cage mate changed daily for 31 days)
Dorsal CA1 of hippocampus on Day 32
Stressed rats
M
M
C 1 G T C A A G G C A G C 2 G T
Average methylation across 12 CG sites within BDNF IV= 25%
Control rats
C 1 G T C A A G G C A G C 2 G T
Average methylation across 12 CG sites within BDNF IV= 5%

FIGURE 5.3

Epigenetic alterations in stressed rats. PTSD-like rats were created using two unpredictable and inescapable 1-hour exposure sessions to a cat. Social instability was also employed, which consisted of changing the animal's cage mate daily for all 31 days of the experiment. Control animals had a stable cage mate throughout the experiment and were never subjected to the cat exposures. As initially reported by Roth et al. [91], bisulfite sequencing PCR revealed significant methylation of BDNF DNA in the hippocampus of stressed rats at the end of the regimen.

stress period. As depicted in Figure 5.3, in our PTSD-like rats at baseline conditions on day 32 of the treatment, we found an increase in methylation of the BDNF gene (exon IV was our target of focus) in the dorsal CA1 [91]. We likewise found increased methylation within the dorsal dentate gyrus (DG) of stressed rats, but a decrease in methylation in ventral CA3.

Epigenetics and cognitive dysfunction

Finally, a third main interpretation regarding the role of epigenetics in the brain is that aberrant epigenetic marking of the genome may serve as a biological basis for cognitive impairment. Two bodies of work that are consistent with this notion are those aimed at understanding the neural underpinnings of schizophrenia and those of the cognitive decline associated with either normal or disease-related aging (i.e., Alzheimer's disease, AD). With regard to schizophrenia, prior to the epigenetics revolution, years of postmortem work and animal model work had long suggested that deficiencies in the extracellular matrix protein reelin and GABA synthesis enzyme GAD_{67} play a significant role in the etiology of schizophrenia. When investigators began examining whether there was a link between epigenetic mechanisms and these events, they found that indeed deficits in reelin and GAD1 (which encodes the GAD_{67} protein) levels paralleled significant methylation alterations within the promoter regions of these genes [92–94]. Genome-wide epigenetic approaches on cortical tissue have now suggested there are many gene loci with altered DNA methylation in schizophrenia, including other gene families related to GABAergic and neurotrophic function [94,95].

With regard to the cognitive decline associated with aging and AD, some very early work in the field showed that there is an age-dependent hypomethylation of the amyloid precursor protein gene [96]. More recently, other studies have emerged showing that age-related epigenetic differences might account for cognitive decline during normal aging. For example, one study assessed histone acetylation changes that are induced by learning in both aged (16 months) and adult (3 months) mice [97]. Several histone posttranslational modifications (PTMs) were examined, and only H4K12 acetylation was absent after learning in the aged animals, specifically in the promoter region of learning and memory-related genes. Not only did treatment with an HDAC inhibitor in the aged group restore their learning deficits, but this group also had H4K12 levels comparable with the adult mice. A second exemplary study examined the DNA methylation of activity-regulated cytoskeleton-associated protein (Arc); the *Arc* gene is important for memory consolidation and synaptic plasticity. Arc transcripts were downregulated in aged rats (24–32 months) compared with adults (9–12 months), accompanied by increased DNA methylation in the promoter region (described in Figure 5.4) of the gene in the hippocampus [98]. In a third study, DNA methylation was found to be increased in aged rats relative to young in CG-dense regions of several neurophysiologically relevant genes whose expression correlates with spatial behavior and decreases with age [99]. Finally, the fourth such study we highlight here utilized a mouse model of AD and showed that increased histone acetylation achieved through HDAC inhibition increased dendrite sprouting and synapse formation and enhanced Morris water maze performance [100]. All of these studies, and others, suggest that the aged brain is characterized by accumulating epigenetic silencing modifications, which inhibit the expression of genes that are important for learning and memory, making the brain less plastic. At the same time, they highlight the point that HDAC inhibitors might be useful enhancers to help ameliorate the effects of aging or AD on cognition.

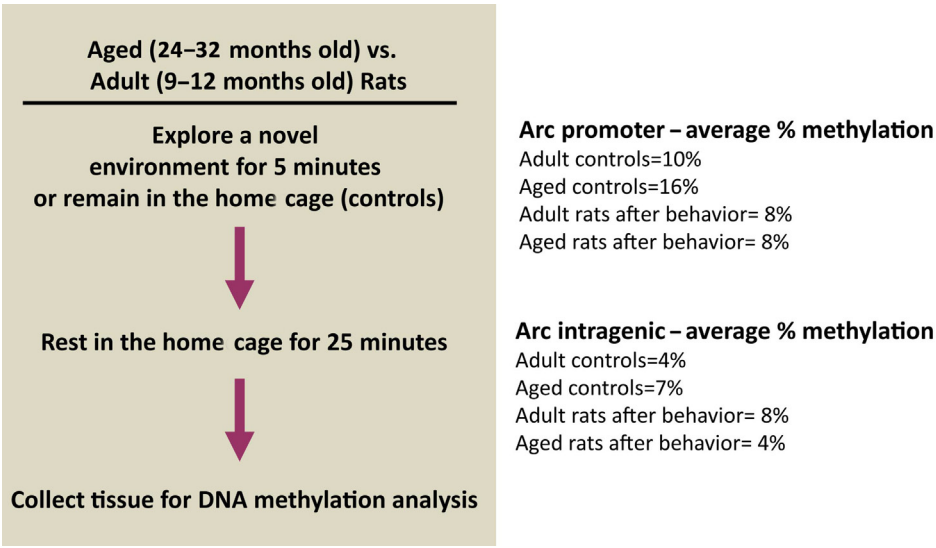
Aged (24–32 months old) vs.
Adult (9–12 months old) Rats
Explore a novel
environment for 5 minutes
or remain in the home cage (controls)
Rest in the home cage for 25 minutes
Collect tissue for DNA methylation analysis
Arc promoter – average % methylation
Adult controls=10%
Aged controls=16%
Adult rats after behavior= 8%
Aged rats after behavior= 8%
Arc intragenic – average % methylation
Adult controls=4%
Aged controls=7%
Adult rats after behavior= 8%
Aged rats after behavior= 4%

FIGURE 5.4

Aberrant hippocampal DNA methylation in aged rats. As described in Penner et al. [98], both aged and adult rats were allowed to explore a novel environment, and patterns of DNA methylation for the *Arc* gene were subsequently assessed in area CA1 of the hippocampus. Age-matched controls remained in the home cage. Aged controls showed significantly more methylation of Arc DNA at the promoter and intragenic regions than did younger rats. Following spatial behavior, both adult and aged rats showed significantly less methylation at the promoter region compared with controls. At the intragenic region, however, adult rats showed a significant increase in methylation following exploration, but aged rats showed a significant decrease.

Concluding remarks

Since the advent of behavioral epigenetics, we have gained fascinating insights into the link between chromatin structure regulation and gene regulation and the brain's embryonic and postnatal development. Moreover, behavioral epigenetics has given us insights into how the brain retains its responsivity (plasticity) to environmental factors after embryonic and early postnatal development. Studies have also revealed that environmental adversity (for example, in the form of social stress or traumatic experiences) can become encoded within epigenetic factors that control gene activity. Together, it has become clear that epigenetic mechanisms operate throughout the lifespan, and even transgenerationally, to influence behavioral trajectories. We certainly still lack a complete understanding of the cause-and-effect role of epigenetic mechanisms in brain evolution, development, and function, but the current view in the field is that further elucidation of the regulatory role of epigenetic processes in aspects of normal and abnormal brain/behavior development will reveal substantial information regarding molecular processes underlying cognition and the susceptibility to (and even resilience for) psychiatric disorders. Importantly, epigenetics now also gives us an avenue to explore new treatment strategies aimed at promoting cognitive and health outcomes.

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Glossary

Astrocytes Specialized star-shaped glial cells involved in many aspects of brain function.
Encephalization An evolutionary increase in the complexity or size of the brain, including a shift of function to cortical regions of the brain.
Imprinted gene Gene in which one copy is epigenetically silenced.
Long-term potentiation Lasting enhancement of signal transmission between neurons facilitating learning and memory.
Monotremes Egg-laying mammals.
Oligodendrocytes Specialized glial cells that wrap around axons.
Synaptic plasticity A change in the efficacy of signaling (a strengthening or weakening) between neurons.
Trier Social Stress Test A laboratory procedure used to induce stress in research participants.
Viviparity Giving birth to offspring that developed within the mother.

CHAPTER

Epigenetic Epidemiology of Psychiatric Disorders

6

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KEY CONCEPTS

- The goal of an epidemiologic study is to use a population-based framework in order to investigate factors that are suspected to be causal in a given disease process.
- Bidirectional influences of epigenetic and environmental factors make it challenging to establish cause versus consequence in epigenetic epidemiology studies of psychiatric disorders.
- A substantial amount of variance in risk remains unaccounted for in most psychiatric disorders, suggesting that well-designed epigenetic studies have much to contribute.

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- The most common study design that has been used to date within psychiatric epigenetic epidemiology is the retrospective case-control design; causal inference can be strengthened in such studies through the inclusion of epigenetic measures from multiple tissues.
- Longitudinal studies remain one of the most preferred study designs for establishing cause versus effect.
- Advantages of this study design include the ability to establish the presence of a disease-associated epigenetic variant prior to disease onset, thus increasing the probability that such variants may be an etiological factor in the disease; however, longitudinal sampling cannot occur for the "target organ" of psychiatric disorders (i.e., the brain).
- A main challenge to conducting epigenetic epidemiologic research is the fact that epigenetic marks are tissue- and cell-type specific. Bioinformatic methods, however, have been developed recently that account for cell-specific epigenetic variation in whole-tissue analyses of DNA methylation.
- We currently have a very limited understanding of the extent of intra- and inter-individual epigenetic variation and the stability of this variation over time.
- Without a better understanding of these parameters, the association of a given epigenetic mark with current or future disease risk (i.e., expected effect size) will be difficult to assess.
- Genome-wide association studies of psychiatric disorders have established standards for establishing positive associations that include controlling for multiple hypothesis testing and replicating positive findings in independent cohorts; similar strategies may eventually be adopted by epigenome-wide association studies of psychiatric disorders when the distribution of population-level epigenetic variation is better understood.
- "We certainly need to remember that between genotype and phenotype, and connecting them to each other, there lies a whole complex of developmental processes. It is convenient to have a name for this complex: 'epigenotype' seems suitable" [1].

ABBREVIATIONS

AD Alzheimer's disease
ALSPAC Avon Longitudinal Study of Parents and Children
ARIES Accessible Resource for Integrated Epigenomics Studies
ASE Allele-specific expression
BD Bipolar disorder
BDNF Brain-derived neurotrophic factor
BPD Borderline personality disorder
CM Childhood maltreatment
COMT Catechol-*O*-methyltransferase
CpG Cytosine–phosphate–guanine
DNAm DNA methylation
ELA Early-life adversity
eQTL Expression quantitative trait loci
EWAS Epigenome-wide association study
GR Glucocorticoid receptor
GREs Glucocorticoid response elements
GWAS Genome-wide association study
HLA Human leukocyte antigen
HPA Hypothalamic–pituitary–adrenal
IGF2 Insulin-like growth factor 2
INPP4A Inositol polyphosphate phosphatase 4A
LCLs Lymphoblastoid cell lines
MB-COMT Membrane-bound isoform of COMT
MDD Major depressive disorder
mQTL Methylation quantitative trait loci
MZ Monozygotic
PTSD Posttraumatic stress disorder
SZ Schizophrenia
SEP Socioeconomic position
SNP Single nucleotide polymorphism

Introduction

Psychiatric disorders are attributable to the combined and interacting influences of heritable factors and environmental influences [2]. Twin studies provide compelling examples of this phenomenon. For example, twin studies show the heritability of schizophrenia (SZ) to be quite high (83%); yet, approximately 17% of the variance in liability to SZ is due to individual-specific environmental effects [3]. Because monozygotic (MZ) twins carry 100% shared genetic information, the unaffected co-twins carry susceptibility genes for SZ but have not expressed the phenotype [3], suggesting the importance of factors beyond DNA sequence variation in determining risk for this disorder. Twin studies of other psychiatric disorders generally show more modest heritabilities, ranging from 31 to 42% for major depressive disorder (MDD) [4], 30 to 40% for posttraumatic stress disorder (PTSD) [5,6], 56 to 86% for anorexia nervosa [7], and 50 to 60% for alcohol dependence [8]. These twin studies suggest a critical effect of genes on psychiatric disorders; however, they also indicate that environmental factors underlie the development of psychopathological phenotypes [9].

But what are the biologic mechanisms that translate environmental exposures into psychiatric disorders? During the past decade, evidence has accumulated that environmental exposures can modify gene expression independently of the primary DNA sequence through epigenetic processes. In the original work by Waddington, who coined the term epigenetics from "epigenesis" and "genetics," epigenetics referred to the interactions of genes with their environment, which then brings the phenotype into being [10–12]. This definition has evolved over time and now generally refers to the stable, but modifiable, regulation of genetic functions that are mediated through non-DNA-encoded mechanisms, including DNA methylation, histone modifications, and non-coding RNAs [13]. A growing literature suggests that epigenetic processes play an important role in psychiatric disorders [14–17]. Nevertheless, there is a relative dearth of research examining how epigenetic marks translate into an increased risk for psychiatric disorders; similarly, it is still quite unknown which epigenetic changes may accompany psychopathological development. The purpose of this chapter is to provide a nonexhaustive overview of the studies to date that have been grounded in epigenetic epidemiology and that have contributed to our understanding of the epigenetic etiology of psychiatric disorders. We will address the challenges associated with causal inference in epigenetic epidemiology, provide specific examples of psychiatric epigenetic epidemiology studies and study designs that have been reported to date, discuss factors that should be considered in designing robust psychiatric epigenetic epidemiology studies, highlight the importance of a life course perspective, and offer an outlook on future research in this burgeoning field.

Causal inference in epigenetic epidemiology

The overall goal in epidemiologic study is to use a population-based framework in order to investigate factors that are suspected to be causal in a given disease process. Although intervention studies do occur within this field, the vast majority of work is based on observational study design, in which causal inference can be a particular challenge due to well-known problems such as confounding and reverse causation [18]. These problems are no less relevant to epigenetic epidemiology, as epigenetic changes may be a consequence rather than a cause of disease [19]. Within psychiatry, the past decade has seen an explosion of research in genome-wide association studies (GWASs), as well as

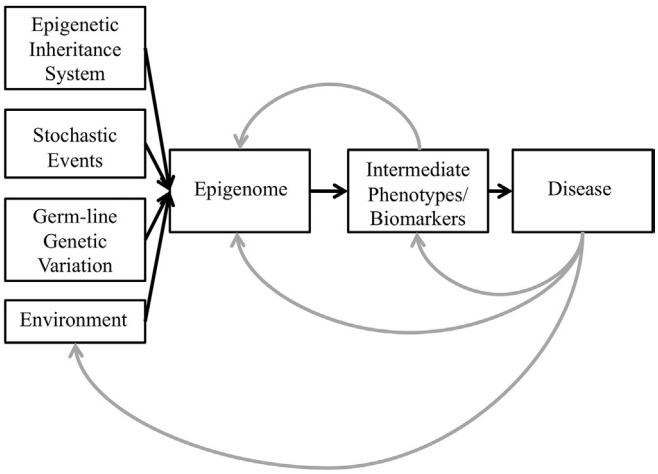

FIGURE 6.1

The complexity of causal inference in epigenetic epidemiology. Multiple factors have been proposed to influence epigenetic patterns, including epigenetic inheritance systems, stochastic events, germ-line genetic variation, and the environment. The epigenome is typically thought to mediate the influence of these factors upon intermediate phenotypes and, subsequently, disease. Straight arrows show simple, unidirectional, linear relationships, whereas the complexities of potential feedback loops are shown by the curved gray arrows. The gray arrows highlight how intermediate phenotypes and/or disease states might impact directly upon the epigenome (reverse causation). In addition, disease might alter the environmental exposure and thus its observed association with the epigenome.

Adapted from Relton CL, Davey Smith, G. Int J Epidemiol 2012; 41: 5–9. With permission of Oxford Journal Press.

candidate gene studies, of psychiatric disorders, motivated in part by the high heritabilities observed across a range of such disorders. A (partial) genetic etiology has been reported for SZ [20], MDD [21], and autism [22], to name a few; furthermore, joint analysis of samples from multiple studies has identified shared genetic liability to multiple psychiatric disorders, including autism spectrum disorder, attention deficit hyperactivity disorder, bipolar disorder, major depressive disorder, and SZ [23]. A common theme across all of these studies is their reliance on very large numbers (i.e., thousands) of cases and controls, and (in most cases) their use of both discovery and replication cohorts. However, because these studies are based on fixed (i.e., germ line) genetic variation, reverse causation and confounding of genetic variation with other exposures are less of an issue [24], although gene–environment correlations should still be considered in any observed associations [25].

In contrast, work in animal models has demonstrated that epigenetic changes occur in response to lived experience and that the resultant epigenetic state influences subsequent physiological reactivity [26]. These bidirectional influences of epigenetics and environment will make it challenging to establish cause vs. consequence in epigenetic epidemiology studies of psychiatric disorders (or, indeed, of any complex disease); these efforts will be further hampered until the nature and extent of epigenetic variation become better characterized in baseline or "control" populations. Figure 6.1 summarizes the challenges to causal inference in this rapidly growing field. Despite the progress made by GWAS

studies, there remains a substantial amount of variance in risk that remains unaccounted for in most psychiatric disorders, suggesting that well-designed epigenetic studies have much to contribute.

Study designs in epigenetic epidemiology

There are many key considerations to designing robust epigenetic epidemiology studies that focus on psychiatric disorders. As discussed above, a key challenge in this field is the establishment of causality; to this end, prospective, longitudinal sampling of study participants prior to disease onset remains a gold standard for identifying epigenetic variants that pre-exist disease and may therefore play a causative role. However, this is not possible to do for the target organ of psychiatric disorders—the brain—and, in addition, is a costly endeavor to undertake. The issue of tissue specificity will be discussed in more detail below. Here, we consider the range of epidemiologic study designs that have been employed to date to study the epigenetic basis of psychiatric disorders, providing examples where appropriate.

Perhaps the most common study design that has been used to date within psychiatric epigenetic epidemiology is the retrospective case-control design. Long a staple of GWASs, as well as other, non-molecular epidemiologic studies, these investigations involve selecting samples from unrelated individuals with known psychiatric diagnoses, as well as a comparable set of controls, both drawn from the same source population. A diverse set of studies, using a range of techniques and testing a variety of tissue types, has been conducted using this type of design. For example, whole blood-based DNA methylation profiles in repetitive DNA sequences in Alzheimer's disease (AD) identified significant elevation of LINE-1, but not Alu or SAT-α, in 43 AD patients versus 38 healthy controls [27]. Similarly, whole blood has been used to compare global methylation levels via high-performance liquid chromatography in SZ patients compared with controls, with results indicating marginally ($p<0.075$) reduced global methylation in 124 male patients versus 108 male controls (but no similar association in females) [28]. Genome-scale case-control designs have also been applied to identify blood-based DNA methylation differences across thousands of genes in association with commonly occurring disorders such as PTSD [29–31], and depression [32], with differences generally identified in genes belonging to biologic pathways previously implicated in these disorders based on non-epigenetic investigations, including immune-related genes in PTSD [29–31] and tryptophan metabolism in depression [32].

Of note, brain-derived samples are well represented in this type of study design, as repeated sampling is not a requirement. For example, candidate gene approaches testing unrelated individuals have identified DNA methylation differences in specific loci known to be important to brain function. The promoter region of the reelin (*RELN*) gene, known to be important for neuronal migration during development, showed significant hypermethylation in postmortem brain tissues obtained from SZ patients in relation to healthy controls [33,34]. Similarly, work investigating the promoter of the membrane-bound isoform of *COMT* determined that this locus is frequently hypomethylated in the frontal lobe of patients with SZ and bipolar disorder (BD), compared with healthy controls. [35] In addition, work on postmortem-derived brain tissues confirms that certain loci (*TrkB*.T1 and the rRNA promoter region) are hypermethylated in suicide completers compared with healthy controls and that this hypermethylation is associated with reduced expression in these genes [36,37]. Complementing these candidate gene examples, approaches that interrogate the entire genome have

reported numerous DNA methylation differences in the frontal cortices of patients with SZ and BD, compared with unaffected controls [38]. Importantly, many of these differences occur in genes that are consistent with current knowledge regarding the neurobiological systems that are involved in major psychosis, including glutamatergic and GABAergic neurotransmission genes and loci involved in neuronal development [38].

For all of these examples, whether from blood or brain, an etiological role cannot be definitively established, as it is not known whether the observed associations occurred prior to disease onset or are a result of the disorder itself. A number of studies, however, have investigated epigenetic variation across multiple tissues, following the premise that similar epigenetic signatures across multiple tissues are more likely to be causative of disease, as they would have had to arise early in development at a time predating disease onset. For example, a focused investigation of DNA methylation in the human leukocyte antigen (HLA) complex group 9 gene (*HCG9*), which had been implicated in psychosis in earlier genome-scale work [38], confirmed that lower DNA methylation at five CpG sites was associated with BD across brain, blood, and germ-line (sperm) tissues (although all three tissues were not necessarily obtained from the same individuals) [39]. Importantly, this work controlled for confounding influences of age and genotype, enabling the authors to detect associations that they might otherwise have missed. Similar, but more limited, work has determined that DNA methylation differences in *COMT* in saliva among SZ and BD patients mirrored that previously observed in the frontal lobe [40]. Note that the genome-scale work described above [38] also included analysis of germ-line (sperm) DNA in a smaller number of psychosis patients versus controls, finding evidence for reduced modularity (i.e., greater between module interference) in both tissues in the patient group. Such multitissue assessments, particularly those that include germ-line tissues, represent a potential strategy that can be used to strengthen the causal inferences of observed associations beyond that of typical case-control studies; ideally, such studies would incorporate multiple tissues sampled from the same case and control individuals.

Another mainstay of epigenetic epidemiology is twin-based studies. Here, MZ twin pairs discordant for the disorder of interest are selected for study: as discussed above, their shared genotype but non-shared phenotype suggests that investigation of non-shared environment and biological factors, including epigenetics, may shed light on the etiology of their disorder. Indeed, some of the earliest human-based work that established a correlation between non-shared environment and epigenetic effects was based on twins' epigenetic profiles, which were shown to be greater among older versus younger MZ twin pairs; notably, twins who reportedly spent less of their lifetime together showed the greatest differences in DNA methylation and histone acetylation [41]. With respect to psychiatric disorders, early twin-based studies adopted candidate gene approaches, identifying, for example, MZ twin DNA methylation differences in lymphocytes at CpG sites in the dopamine D2 receptor gene (*DRD2*) [42]; here, the "epigenetic distance" between unrelated, affected twins was determined to be less than that between the affected and unaffected co-twins. More recent studies have also adopted candidate gene approaches, examining the serotonin transporter (*SLC6A4*) locus in leukocyte-derived DNA among twin pairs discordant for MDD and PTSD, finding that within-pair differences in DNA methylation at 10–20 CpG sites examined correlated significantly with within-pair differences in depression scores; this association remained after adjustment for potential confounders [43]. The development of genome-scale approaches has enabled more recent twin-based studies to include broader assessments of the epigenetic etiology of psychiatric disorders. Interestingly, one of these genome-scale twin studies ultimately identified DNA methylation differences in *SLC6A4*, in DNA

derived from lymphoblastoid cell lines (LCLs), as a potential etiological factor in BD [44]; although this initial result was based on only two twin pairs, the findings were supported by subsequent case-control analysis of both LCLs and frontal cortices in unrelated BD and control individuals, reported in the same study [44]. In addition, a recent genome-scale study of 22 MZ twin pairs discordant for SZ and BD identified eight loci with psychosis-related differential methylation in DNA derived from whole blood [45], with the top-ranked differentially methylated locus showing a relative decrease in methylation in psychosis cases compared with controls, a pattern that was also observed (although somewhat attenuated) in postmortem brain samples. One important finding from this genome-scale work is that there was substantial heterogeneity in the intra-pair differences in DNA methylation among even the top-ranked differentially methylated loci, with a wide range of methylation differences observed within each twin pair. These results highlight an important issue within epigenetic epidemiology, namely that there is currently no established range of expected effect sizes for epigenetic differences associated with psychiatric disorders. Nevertheless, as the authors note [45], this observed heterogeneity is generally consistent with the hypothesis of rare etiological factors of large effect that may play a role in some cases of psychiatric disorders.

The challenges of causal inference in epidemiology make longitudinal studies one of the most preferred study designs for establishing cause versus effect, and this is no less true of epigenetic epidemiology. As discussed above, advantages of this study design include the ability to establish the presence of a disease-associated epigenetic variant prior to disease onset, thus increasing the probability that such variants may be an etiological factor in the disease. The disadvantages include the high cost of establishing such studies within a population-based framework, the length of time it requires to collect data that address one's primary hypothesis, and—for psychiatric disorders—the inability to obtain samples from the target organ of disease. Despite these hurdles, examples do exist of longitudinal, population-based epigenetic studies of psychiatric disorders. To date, these have focused largely on identifying epigenetic variants associated with PTSD, drawing on biobanked samples from individuals enlisted in the U.S. military. For example, work on serum samples obtained from U.S. military service members pre- and postdeployment identified increased DNA methylation in LINE-1 repetitive sequences post- versus predeployment, but only among service members without PTSD. Also, relatively higher DNA methylation was observed in *Alu* repetitive sequences in pretrauma PTSD cases versus controls prior to deployment [46]. This latter finding is especially noteworthy as it existed prior to putative trauma exposure and may thus represent a pre-existing vulnerability factor for PTSD. Additional work by the same group of researchers investigated DNA methylation levels in the promoter regions of five genes previously implicated in PTSD etiology, including *IGF2*, *H19*, *IL8*, *IL16*, and *IL18*. Significant changes across deployment between cases and controls were identified, again in serum-derived DNA, for two of the genes, *H19* and *IL18*; differences in the former were driven by reductions of DNA methylation across deployment in controls only, whereas in the latter gene the authors observed an increase in DNA methylation in cases, but a reduction in controls, across deployment. Furthermore, within *IL18*, the authors also observed significantly lower DNA methylation predeployment among cases versus controls at three of the five CpG sites tested, as well as for the mean of all CpG sites tested within this gene [47]. As with the earlier *Alu*-based findings [46], these pre-exposure differences in *IL18* again suggest a potential vulnerability factor with respect to risk for PTSD.

Of special consideration in longitudinal studies is the possibility of assessing the effects of an intervention. Measures can be obtained both prior to and following the application of an intervention

within a sample of individuals suffering from a given disease and controls, in order to assess the effects of the intervention on epigenetic patterns. As with the more general, non-intervention longitudinal studies, to date there have been few studies reporting results from this study design. Work focusing on the dopamine transporter gene (*SLC6A3*) in a sample of alcohol-dependent patients found no difference in DNA methylation levels in whole blood when compared with controls upon admission to a hospital-based, 14-day detoxification period (i.e., at baseline) [48]. The 14-day detoxification period included cognitive behavioral therapy and the potential use of benzodiazepines if deemed necessary; although no significant change was observed between *SLC6A3* DNA methylation levels in patients at baseline and after intervention, repeated measures analysis of patients showed evidence of a significant association between age and *SLC6A3* DNA methylation, a finding that was absent in analyses of controls. Because the age-associated findings were identified in the patient group only, the authors suggest that it is likely a consequence, rather than a cause, of alcohol-use disorder [48].

In contrast, work focusing on the brain-derived neurotrophic factor (*BDNF*) locus identified psychotherapy-associated DNA methylation changes in whole blood among study participants with borderline personality disorder (BPD) [49]. Compared with controls, BPD showed significantly higher DNA methylation levels in two exons of the *BDNF* locus at baseline; this finding is likely due to the higher exposure to childhood maltreatment experienced by the patient group, as linear regression analyses demonstrated a strong association between increased childhood trauma exposure and higher *BDNF* methylation [49]. Over 4 weeks of treatment, however, there was a significant decrease in depression severity, hopelessness, and impulsivity among BPD subjects following psychotherapy. The associations with *BDNF* methylation were not straightforward, however, as *BDNF* methylation increased significantly over time in the entire patient group, due mainly to the changes observed in patients responding poorly to psychotherapy. Specifically, patients with $\leq$50% improvement in depression scores over the treatment period showed increases in *BDNF* DNA methylation; in contrast, those with >75% improvement in depression showed significant decreases in *BDNF* DNA methylation levels [49]. Similar patterns were seen in association with improvements in hopelessness and impulsivity, although the decreases in the responders did not reach statistical significance [49]. The heterogeneity in results according to response-to-treatment are in line with earlier work on *BDNF* which demonstrated that, overall, 8 weeks of treatment with citalopram was associated with a reduction of H3K27 histone methylation and an increase in gene expression in blood among 25 patients suffering from MDD; however, subgroup analyses of responders and non-responders showed significant changes in the responders only, defined as those with Hamilton Rating Scale for Depression scores less than 9 [50]. Although this work did not include control participants in its study design, earlier work by the same group had demonstrated that postmortem prefrontal cortex tissue from subjects with versus without antidepressant treatment and from controls was associated with the same effects reported in blood; that is, H3K27 trimethylation levels in *BDNF* were lower in those treated with antidepressants compared with non-treated depressed subjects and with controls [51]. These intervention studies, while currently few in number, highlight the potential of developing epigenetic biomarkers of treatment response for both pharmacotherapeutic and psychotherapeutic interventions.

The study designs discussed above represent the vast majority of epigenetic epidemiologic study designs applied to date with relevance to psychiatric disorders; the relevant studies are summarized in Table 6.1. Several additional study designs pertinent to epigenetic epidemiology exist, however, and although these have yet to be applied extensively to psychiatric outcomes, their application to these ends stands to increase in the coming years. For example, studies based on human subjects are

Table 6.1 Summary of Epigenetic Epidemiology Study Designs and Their Relevant Findings

Study Design	Sample Size	Tissue Type	Disorder Assessed	Main Finding	Reference
	81	Whole blood	Alzheimer's disease	Alzheimer's participants exhibited increased LINE-1 DNAm compared with healthy controls.	Bollati et al. [27]
	447	Peripheral leukocytes	Schizophrenia	Marginally lower global DNAm in male patients vs. male controls was observed; however; no difference was seen in female participants.	Shimabukuro et al. [28]
	396	Peripheral blood	PTSD	Global DNAm and gene expression profiles reveal overlapping networks regarding cell survival, cell development, cell migration, cell adhesion, and T-cell activation, immunity networks. Non-overlapping networks were also seen.	Mehta et al. [29]
	110	Whole blood	PTSD	Increased DNAm in PTSD participants was observed globally. CpG siltes in *TPR, CLEC9A, APC5, ANX2,* and *TLR8* were differentially methylated in PTSD.	Smith et al. [30]
	100	Whole blood	PTSD	Unmethylated genes associated with PTSD are enriched for immune function. Methylation levels of genes associated with traumatic burden are also enriched for immune function.	Uddin [31]
	100	Whole blood	Depression	Increased DNAm (brain development and tryptophan metabolism) and decreased DNAm (lipoproteins) specific to participants with depression.	Uddin [32]
	10	Postmortem frontal lobe	Schizophrenia	DNAm of the *RELN* promoter region is significantly hypermethylated in schizophrenia patients. Inverse relation between *RELN* DNAm and mRNA gene expression.	Abdolmaleky [33]

(*Continued*)

Table 6.1 (Continued)

Study Design	Sample Size	Tissue Type	Disorder Assessed	Main Finding	Reference
Retrospective case-control	30	Postmortem cortex	Schizophrenia	Hypermethylation of *RELN* DNAm is associated with schizophrenia.	Grayson [34]
	115	Postmortem frontal lobe	Schizophrenia & bipolar disorder	Hypomethylation of *MB-COMT* is common in schizophrenia and bipolar disorder compared with controls; likewise, mRNA levels show an accompanying increase in transcript levels of *MB-COMT*.	Abdolmaleky [35]
	30	Postmortem brain	Suicide	Significant hypermethylation of rRNA promoter and 5' region in the hippocampus—but not cerebellum—of suicide victims, which is consistent with reduced rRNA expression in the hippocampus.	McGowan [36]
	30	Postmortem brain	Suicide	Histone methylation of *TrkB.TI* is increased at lysine 27 in the orbital frontal cortex—but not cerebellum—in suicide completers compared with controls and is associated with reduced gene expression of the gene.	Ernst et al. [37]
	134	Postmortem cortex and sperm	Schizophrenia & bipolar disorder	Psychosis-associated DNAm differences occur in numerous processes related to disease etiology, including glutamatergic and GABAerigic neurotransmission and brain development. Decreased epigenetic modularity in both the brain and germ line of patients suggests that systemic epigenetic dysfunction is associated with major psychosis.	Mill et al. [38]
	1402	Postmortem brain, peripheral blood, sperm	Bipolar disorder	Decreased *HCG9* DNAm was associated with bipolar disorder across postmortem brain, blood, and sperm.	Kaminsky [39]
	85	Saliva	Schizophrenia & bipolar disorder	Hypomethylated *MB-COMT* promoter region DNAm in schizophrenia and bipolar participants compared with healthy controls.	Nohesara [40]

	4	Lymphocytes	Schizophrenia	The “epigenetic distance” between discordant co-twins was larger than between the affected discordant twin and the concordantly affected twins.	Petronis [42]
	84	Peripheral blood leukocytes	Depression	Intrapair differences in DNAm at 10 of 20 CpG sites in *SLC6A4* promoter were significantly correlated with intrapair differences in the BDI scores.	Zhao et al. [43]
Twin studies	4 (103 case-control)	Lympoblastoid cell line & postmortem brain	Bipolar disorder	Hypermethylation of *SLC6A4* in one of two affected twins. DNAm was significantly associated with mRNA expression levels in participants with the SS genotype; expression levels were reduced in participants with bipolar disorder; and postmortem brain showed increased DNAm at same site as LCLs in BD patients.	Sugawara [44]
	44 (45 case-control)	Peripheral blood and postmortem brain	Schizophrenia & bipolar disorder	Multiple DNAm differences observed among those with psychosis, schizophrenia and bipolar disorder. The top psychosis-associated, differentially methylated region was in the promoter of *ST6GALANC1*, showing significant hypomethylation in affected twin; however, the direction of effect varied among families. A subset of postmortem brains from psychosis patients showed similar patterns of decreased DNAm at the *ST6GALANC1* locus.	Dempster et al. [45]
Longitudinal studies	150	Serum	PTSD	Hypermethylated LINE-1 DNAm in non-PTSD controls post- vs. predeployment; hypomethylated LINE-1 in cases vs. controls postdeployment. *Alu* was hypermethylated in cases vs. controls predeployment.	Rusiecki et al. [46]

(*Continued*)

Table 6.1 (Continued)

Study Design	Sample Size	Tissue Type	Disorder Assessed	Main Finding	Reference
	150	Serum	PTSD	Significant decreases in *H19* and *IL18* DNAm in controls across deployment. Increase in *IL18* DNAm in PTSD cases. Significantly reduced in *IL18* DNAm in cases vs. controls predeployment at 3 of 5 CpGs tested and in the average of all sites.	Rusiecki et al. [47]
	200	Whole blood	Alcohol dependence	No significant change was observed in *SLC6A4* DNAm patients during the intervention period. Significant increase in DNAm with age in patients.	Nieratschker et al. [48]
	167	Peripheral blood leukocytes	Borderline personality disorder	Significantly higher DNAm in exon I and IV in *BDNF* associated in patients vs. controls. DNAm in *BDNF* increased after intensive dialectical behavior therapy; however, participants who responded to therapy showed a decrease in *BDNF* DNAm whereas non-responders exhibited an increase in DNAm.	Perroud et al. [49]
Intervention studies	25	Peripheral blood	Major depressive disorder	A decrease in H3K27 histone methylation was observed after 8 weeks of treatment with Citralopram in MDD patients, accompanied by an increase in *BDNF* expression. Significant changes in methylation driven by patients responsive to treatment when examining HAM-D scores.	Lopez et al. [50]
	27	Post-mortem prefrontal cortex	Major depressive disorder	Participants treated with antidepressants possessed significantly lower H3K27 methylation levels compared with non-treated depressed participants and controls.	Chen et al. [51]

appropriately regulated by rules that substantially limit the conditions under which experimentation with such subjects may occur. "Natural experiments," however, can form the basis of studies based on human subjects for which the exposure was not under investigator control. A well-known example comes from the Dutch Hunger Winter Families study, in which people residing in German-occupied portions of the Netherlands were subjected to famine in the winter of 1944–45 due to a German-imposed food embargo during the end of World War II. These conditions enabled researchers, decades later, to examine the effects of malnutrition on human development by examining the effects of famine on surviving offspring of women who were pregnant at the time. Epidemiologic studies of this "natural experiment" reported that individuals who were prenatally exposed to famine during the Dutch Hunger Winter showed a twofold increase in the cumulative risk of SZ [52,53]. Although these observations did not include epigenetic measures, subsequent studies based on the Dutch Hunger Winter Families cohort found that prenatal exposure to famine—specifically periconceptual exposure—was associated with reduced methylation of insulin-like growth factor 2 (somatomedin A) (*IGF2*) six decades after famine exposure [54]. The *IGF2* gene is an imprinted locus and plays a key role in the regulation of cellular proliferation and growth [55]. Postmortem studies indicate that brain weight in males is positively correlated with DNA methylation at *IGF2* [56] and, in turn, that decreased brain weight is associated with SZ [57]. The dual association of reduced *IGF2* methylation with two factors previously associated with SZ—exposure to famine and lower brain weight—renders it plausible that epigenetic mechanisms may mediate the effect of developmentally sensitive environmental exposures on the onset of this psychiatric disorder. Such inferences would be strengthened by future investigations that explicitly link natural experimental exposures, epigenetic variation, and psychiatric outcomes within the same study design.

An additional study design that stands to make large contributions to epigenetic epidemiologic studies of psychiatric disorders is birth cohorts. In these studies, data are typically collected from pregnancy onward, in order to capture the exposures of interest as early in development as possible (i.e., beginning *in utero*) and their potential effects on epigenetic variation. Biospecimens may be collected both during pregnancy and postnatally; alternatively, specimen collection may begin postnatally only. Examples of studies with a birth cohort design include the British Birth Cohort, which has followed study participants from 1958 onward. The original cohort was based on a sample of over 17,000 individuals born in England, Scotland, and Wales during one week in the year 1958 [58]. Comprehensive health and medical data have been collected from study participants at regular intervals since this time; from 2002 to 2004, blood samples were collected from consenting study participants that enables epigenetic testing. Recent work on a subset of samples from adult males in this cohort demonstrated a greater number of differentially methylated genes associated with childhood versus adulthood socioeconomic position (SEP) [59], a finding consistent with earlier work (in an independent cohort) based on gene expression signatures [60]. Although, to our knowledge, similar work has yet to be reported linking early-life SEP, epigenetics, and psychiatric disorders, population-based studies in the United States [61] and United Kingdom [62] have provided evidence of the adverse effects of early-life SEP on later-life mental illness. Data collected from birth cohorts stand to provide a crucial window into the possible mediating mechanisms of this well-established relationship. Resources such as the Accessible Resource for Integrated Epigenomics Studies (ARIES) are particularly valuable in this regard, due to their goal of generating integrated epigenetic, genetic, and transcriptomic data from multiple tissues and time points across the life course, along with fine-grained anthropometric, health, and behavioral data collected from 1000 mother/child pairs from the

Avon Longitudinal Study of Parents and Children (ALSPAC) [63]. Of note, the ALSPAC study holds further promise in that fathers and siblings are now being assessed in addition to mother/child pairs, providing the possibility of conducting a family-based study design of epigenetic factors associated with psychiatric disorders, a study design not yet widely employed in this field of research.

Additional promise within the realm of birth cohorts comes from biospecimens that are banked at birth from newborns. Here, specimens are collected routinely, often as a part of newborn screening programs, and are subsequently retained for potential research use. In the United States, for example, laws regarding the retention and use of neonatal dried blood samples existed in 20 states as of 2011, and in four of these states the samples become property of the state [64]. To fully leverage this resource in the context of psychiatric disorders, samples must be associated with appropriate phenotype data (and possibly additional biologic data as well), which can be collected from consenting participants in follow-up studies. In certain countries, such as Norway [65], efforts are underway to enable such specimens to be linked to health registries so that rapid phenotyping may be conducted without additional contact with the subject.

Methodologic issues to consider in epigenetic epidemiology

The above section provided a general overview to the main study designs currently employed in epidemiologic studies of psychiatric epigenetics. Many of the issues described pertain to epidemiology more generally. In the next section, we provide a brief overview of some methodological considerations that are largely unique to epigenetic epidemiology.

Tissue and cell specificity of epigenetic marks

One of the main challenges to conducting epigenetic epidemiologic research is the fact that epigenetic marks are tissue- and cell-type specific. Early work profiling portions of the human genome identified tissue-level specificity of DNA methylation across 12 tissue types [66], a finding that has since been replicated across the entire genome for a large number of tissues [67]. Furthermore, even within a specific tissue type (e.g., whole blood), cell-specific epigenetic profiles suggest that measuring average epigenetic levels within a tissue may miss important, cell-specific variation [68] that could contribute to disease risk. Although this issue is not unique to epigenetic measures—transcriptional activity, for example, is known to vary by tissue and cell type as well—it poses a particular problem for epigenetic epidemiology studies, where conducting fine-scale studies of cell-specific epigenetic marks is not feasible within a population context. In addition, many cohort studies on which population-based psychiatric epigenetic research might be conducted include banked samples of whole blood, blood spots, serum, or saliva, each of which contains DNA from multiple cell types; thus, if the full potential of these valuable cohort studies is to be realized, methods to account for cell-specific epigenetic variation are clearly required.

Fortunately, bioinformatic methods have been developed recently that account for cell-specific epigenetic variation in whole-tissue analyses of DNA methylation. Specifically, Houseman and colleagues [69] developed an analytic strategy based on regression calibration, which considers a DNA methylation signature to be a correlated, but imperfect, measure of leukocyte distribution—in other words, a high-dimensional multivariate surrogate for the distribution of white blood cells.

The method itself relies on the concept of differentially methylated regions as markers of immune cell identity using a DNA methylation microarray platform, which can then be used to estimate the proportion of immune cells in unfractionated whole blood and, importantly for population-based research, does not require fresh cells to apply the algorithm [69]. Within the original paper, the authors validated their approach using 46 white blood cell samples, subsequently predicted leukocyte distributions in cell mixture experiments of 12 blood cell samples, and applied the method to DNA methylation datasets from multiple studies, including ovarian cancer, obesity, and Down's syndrome-based datasets. This method has since been validated using an independent dataset [70]. Although, at the time of writing, the method has not been applied in the context of psychiatric disorders, it has been taken up by other subfields of epigenetic epidemiology, including epigenome-wide association studies of arthritis [19] and smoking [71]. The former study was able to confirm the estimated cell proportions with flow cytometric data on a subset of participants, with results showing that there were significant cellular differences among rheumatoid arthritis cases (e.g., decreased proportion of T-cells, increased proportion of granulocytes) versus controls [19]. The latter study found the top-ranked CpG sites significantly associated with smoking after adjustment of the proportion of leukocyte subsets [71]. Application of this approach thus holds promise for future DNA methylation-based studies of psychiatric disorders that are based on whole blood—a tissue type that is readily accessible for longitudinal studies. Similar approaches have been developed for reducing the effects of cellular heterogeneity in the brain. Here, Guintivano and colleagues [72] used postmortem frontal cortex samples from 29 cases with MDD and 29 matched controls to identify cell-type-specific DNA methylation variation that distinguished neuronal versus glial cell types. Their resulting bioinformatic tool is freely available and can be used to remove cell-type heterogeneity bias from DNA methylation profiles derived from bulk tissue preparations generated on Illumina® microarrays [72]. Although this approach is relevant only to epigenetic studies involving deceased individuals, the pairing of more precise postmortem, brain-based analyses with prospectively acquired samples from peripheral tissues (or cross-sectional studies sampling multiple tissues within the same individual) stands to make important contributions toward clarifying the epigenetic etiology of psychiatric disorders.

In addition to cellular heterogeneity within a single tissue, an important further consideration in designing epidemiologic research is selecting which tissue to study. As discussed above, this is particularly challenging for psychiatric disorders, where the tissue of interest is not accessible to sampling from living individuals. Longitudinal studies—ideal for inferring causation—are thus limited to assessing epigenetic marks in peripheral tissues. To date, the largest proportion of psychiatric epigenetic studies conducted within a population-based framework has relied upon blood-derived tissues as the tissue of choice (e.g., 30–32,46,47). A more limited number of studies have used LCLs (e.g., 73,74), saliva [40], and buccal samples (e.g., 75) to obtain DNA for epigenetic profiling. Although these tissues may serve as valuable biomarkers of disease onset and/or progression, the question of which tissue is most likely to reflect the organ of interest—the brain—remains somewhat unresolved. Recent work comparing the utility of buccal versus blood cells as surrogate tissue for epigenome-wide association studies (EWASs) found initial compelling evidence that buccal cells may be more informative than blood [76]. Specifically, buccal cells were found to be significantly hypomethylated compared with blood; this is an important distinction, as DNaseI hypersensitive sites are a strong predictor of regulatory activity and are often observed at low methylated regions. Comparative analysis with ENCODE DNaseI hotspots common across all epithelial cells (from which buccal cells are derived) versus those common across all blood subsets revealed significantly more hotspots in the

former tissue, suggesting that buccal cells are characterized by significantly more active regulatory elements than blood [76]. The authors further assessed the relevance of blood versus buccal tissue to disease state by performing spatial correlations of tissue-specific hypomethylated sites with statistically significant single nucleotide polymorphisms (SNPs) from published GWAS studies. Although hypomethylated sites in blood were found to be strongly associated with a variety of autoimmune diseases/phenotypes, those in buccal tissue were associated with diseases and/or phenotypes associated with epithelial and not just buccal function, such as bladder cancer [76]. Furthermore, there was greater overlap in hypomethylated sites between buccal and other tissues than there was between blood and other tissues overlap. In addition, unsupervised clustering of different tissues of all probes on the Illumina® 450K array demonstrated that blood samples clustered together to the exclusion of all other tissues, including buccal, stem cells, brain, kidney, and sperm [76]. Taken together, these results suggest that inclusion of buccal cells in epigenetic epidemiology studies stands to enhance EWAS investigations of non-blood-based diseases, such as psychiatric disorders.

If resources are not too limited, another possible strategy is to sample tissues representing the three germ layers, as previously suggested [77]. For example, collection from the mesoderm could include samples of blood, biopsies of skeletal muscle, subcutaneous fat, and the dermal layer of a skin punch biopsy (i.e., fibroblasts); collection from the ectoderm could include samples of the epidermal layer of a skin punch biopsy (i.e., keratinocytes) and buccal cells; and collection from the endoderm could include urine samples (reflective of the bladder lining) and stool samples (reflecting colonic mucosa) [77]. These approaches would allow investigators to assess concordance of epigenetic marks among tissues within individuals over time, with and without the disease state of interest. This could be complemented by publicly available resources, such as the Roadmap Epigenomics Project [78], through which it is now feasible to infer, in general, the concordance of epigenetic marks across tissues; similarly, the University of California Santa Cruz (UCSC) Genome Browser currently displays ENCODE [79] tracks of DNA methylation and histone modifications across multiple cell types, enabling an overview of epigenetic variation in these cell types across the human genome.

Defining mechanistic relationships among DNA sequence, epigenetic, and gene expression variation

The focus of this chapter has been to review the current state of epigenetic epidemiology studies of psychiatric disorders. In order to fully appreciate the potential of such epigenetic studies, however, consideration must be given to additional molecular marks that interact with epigenetic variation. As a key regulator of gene expression, epigenetic factors play an important role in determining gene function. However, epigenetic factors themselves are known to be affected by underlying DNA sequence variation; thus, in order to comprehensively understand etiologic pathways to complex diseases such as psychiatric disorders, careful attention should be given to all three layers of genomic information, with integration across layers pursued in studies where possible. A major challenge in this regard is that we currently have a fairly poor understanding of the mechanistic relationship among DNA sequence variation, epigenetics, and transcription; furthermore, it is not yet clear if changes in epigenetic factors such as DNA methylation cause changes in gene expression, or whether such changes in such factors may result from changes in gene expression.

To address this issue, a recent study investigated DNA sequence variation, transcription levels, and DNA methylation variation across three cell types derived from umbilical cord samples in 195

individuals, including fibroblasts, T-cells, and immortalized cells from LCLs [80]. Among the many important—and some surprising—findings from this work, DNA methylation sites showed negative correlations with gene expression across genes, independent of whether they correlated positively or negatively with gene expression across individuals. As noted by the authors, these findings suggest that the mechanisms contributing to inter-individual DNA methylation variation that associates with gene expression is at least partially distinct from the mechanisms involved in the establishment of repressive promoter DNA methylation marks set down across genes during development [80]. Furthermore, the authors found that allele-specific expression (ASE) was driven primarily by DNA sequence variation; that is, there was no evidence for DNA methylation alone contributing to ASE (except for in imprinted genes), suggesting that hemimethylation is an infrequent occurrence. To further dissect causative relationships among the three molecular measures, the authors assessed, in each cell line, the likelihood of an SNP independently affecting gene expression and DNA methylation (thus indicating a passive role for methylation with respect to gene expression); an SNP affecting methylation, which then affects expression (active role); and an SNP affecting gene expression, with expression then affecting methylation (again indicating a passive role for methylation). Importantly, all three scenarios were observed to occur, with the relative frequency of each scenario differing by cell type. The independent model was the most likely in fibroblasts and lymphoblastoid cells; however, in T-cells, the most likely model was one in which DNA methylation takes an active role in shaping gene expression. These results highlight the complex, tissue-specific relationships among DNA sequence, epigenetic, and gene expression variation that are only beginning to be understood. As the number of positive reports of epigenetic associations with complex disease continues to grow, it will be especially important to identify those associations that cause gene expression changes and those that are only passively linked to such changes, in order to more precisely define true biomarkers of disease and/or possible targets for pharmaceutical interventions [81].

Specific investigations of the effects of DNA sequence, epigenetic, and gene expression variation on psychiatric disorders within a single study are not yet prevalent in psychiatry. Nevertheless, recent work has adopted a functional genomics approach to studying BD. Here, investigators focused on exploring the effects of DNA sequence variation previously associated with BD in earlier GWASs on DNA methylation and transcription in the cerebellum, in order to gain an improved understanding of the functional consequences of these variants on risk for BD. SNPs previously associated with BD in the Wellcome Trust Case Control Consortium were found to be enriched for methylation quantitative trait loci (mQTL) in cerebellum-derived DNA occurring in *cis* (i.e., within 4MB) to the implicated SNP [82]. The study also found an excess of expression quantitative trait loci (eQTL) in the top bipolar-associated SNPs, again in *cis*. Although only eight mQTL–eQTL pairs were identified that shared the same SNP gene combination—a result that is not greater than that expected by chance—SNPs affecting both DNA methylation and gene expression where the target genes are allowed to differ were significantly enriched among the top BD-associated SNPs [82], suggesting that these molecular features should be considered separately in terms of their biological function. Interestingly, the authors also sought to test whether incorporating mQTL information into GWASs could enhance power to detect significant associations. By restricting their association analyses to the *cis* mQTLs, the authors identified one SNP in the inositol polyphosphate phosphatase 4A (*INPP4A*) locus, which locally regulates methylation in this gene and is associated with bipolar disorder at a level that survives stringent Bonferroni correction [82]. Notably, this gene had been previously implicated in BD [83]. This study represents the potential of an integrated functional genomics approach to more precisely identify functionally important epigenetic variation of relevance to psychiatric disorders.

Unknown effect sizes in disease-associated epigenetic variation

The current state of epigenetic epidemiology has been compared by some to be similar to the early days of GWASs, in which much initial research was conducted without a full appreciation of the expected strength of association between a particular SNP and a complex phenotype. As discussed elsewhere in this chapter, the situation for epigenetic epidemiology is complicated by challenges to causal inference, given the potential changes to epigenetic factors over time, their tissue specificity, and, for psychiatric epigenetic epidemiology at least, the inaccessibility of the target tissue to longitudinal sampling. A further important consideration that *is* similar to the early days of GWASs, however, is our current very limited understanding of the extent of intra- and inter-individual epigenetic variation; and – unique to epigenetic studies – the stability of this variation over time. Without a better understanding of these parameters, the association of a given epigenetic mark with current or future disease risk (i.e., expected effect size) will be difficult to assess. Whereas global DNA methylation has been shown to change over time [84], epigenetic changes in specific loci can be more stable. For example, a focused study of intra-individual change in DNA methylation in both blood and buccal showed that five of eight loci tested (*IGF2R*, *LEP*, *CRH*, *IGF2*, and *APOC1*) demonstrated relative stability over time ($\rho > 0.75$) in both tissues [77]. In contrast, several loci of interest to psychiatric researchers (*DRD4*, *SLC6A4, MAOA*) showed poor stability in DNA methylation levels over time within individuals between 5 and 10 years of age (although *DRD4* showed relatively high inter-individual stability over this time period) [85]. Table 6.2 provides a summary of age-related effect sizes in these and selected additional studies.

Given that many of the disease-associated DNA methylation differences currently identified are characterized by small absolute changes (<5%) [87], it is particularly critical to delineate the extent of intra- and inter-individual epigenetic variability of putative disease-associated loci. Ideally, loci targeted for testing in epigenetic epidemiology studies should demonstrate inter-individual variation in DNA methylation, stability of this variation in DNA methylation over time, and—if identifying causal epigenetic variants is the goal (rather than, say, identifying a biomarker of disease)—a correlation between DNA methylation as measured in blood and the tissue of interest [77]. Moving forward, resources such as the previously mentioned epigenomics roadmap, augmented by additional databases of epigenetic variation observed in psychiatric disorders across the genome, will be invaluable for establishing expected ranges of epigenetic variation in normal and diseased states. Similarly, systematic epigenomic equivalents of GWASs—EWASs—should do much to bridge our current gap in knowledge regarding the extent of intra- and inter-individual epigenetic variation [88] and help to develop more streamlined protocols for epigenetic epidemiology studies akin to those currently in play for GWAS investigations.

Importance of a life course perspective

As discussed above, epigenetic epidemiology studies based on birth cohort designs stand to make particularly important contributions to our understanding of complex disease. With respect to psychiatric disorders, a wealth of evidence has established that such early-life experiences (in particular, adverse early-life experiences) increase the prevalence of psychiatric disorders later in life [89–91], suggesting a biologic pathway whereby exposure to early-life adversity (ELA) physically manifests and later contributes to the increased prevalence of mental illness. However, the epigenetic factors that may contribute to the link between ELA and subsequent psychiatric disorder remain poorly understood, despite evidence of their likely importance in BPD [49,92], depression, and PTSD [29].

Table 6.2 Summary of Select Studies Examining Intra-Individual Change in DNA Methylation Over Time (i.e., Age-Related DNA Methylation Changes) and Their Effect Sizes

Tissue	Variable	Findings	Locus/DNAm Measure	Effect Size Range	Reference
Peripheral blood	Age	Significant ($p < 0.001$) decrease and increase in global DNAm with age, suggesting a loss of epigenetic patterns with age. Also reporting familial association with epigenetic patterns.	Global DNAm	DNAm change ranged from −30 to + 26% over 11 years in one cohort, and between −49% to +39% over 16 years in a second cohort. Difference in DNAm over time showed familial clustering patterns: in the family with the greatest change in global DNAm, the top gene showing age-related DNAm differences was *PWCR1*, with a loss of DNAm of 72.3% over an average of 16 years.	Bjornsson et al. [84]
Buccal cells	Age	Low intra-individual correlation ($p > 0.05$) in DNAm change in ages 5 to 10 years. Data suggest DZ twins DNAm change in opposite directions over time, while MZ twins change in the same direction.	*MAOA*	MZ: $r = 0.35$, $p = 0.16$; DZ: $r = -0.15$, $p = 0.53$. Male DNAm change ranged from −15% to +24%; Female DNAm change ranged from –34% to +58%.	Wong et al. [85]
		Low intra-individual correlation ($p > 0.05$) in DNAm change in ages 5 to 10 years.	*SERT*	MZ: $r = 0.22$, $p = 0.17$; DZ: $r = 0.15$, $p = 0.39$. DNAm change ranged from –41% to +23%.	
		Significant ($p < 0.001$) intra-individual change between the ages of 5 to 10 years.	*DRD4*	MZ: $r = 0.52$, $p < 0.001$; DZ: $r = 0.45$, $p = 0.003$. DNAm change ranged from –19% to +21%.	

(*Continued*)

Table 6.2 (Continued)

Tissue	Variable	Findings	Locus/DNAm Measure	Effect Size Range	Reference
Whole blood	Age	Increase in DNAm over an 11–20 year interval	*IL10*	$\rho = 0.422$. DNAm change over time 2.8% ± 9.1.	Talens et al. [77]
		Increase in DNAm over an 11–20 year interval	*IGF2R*	$\rho = 0.883$. DNAm change over time 1.8% ± 8.1.	
		Increase in DNAm over an 11–20 year interval	*LEP*	$\rho = 0.895$. DNAm change over time 1.8% ± 6.1.	
		Increase in DNAm over an 11–20 year interval	*CRH*	$\rho = 0.942$. DNAm change over time 0.4% ± 6.7.	
		Decrease in DNAm over an 11–20 year interval	*IGF2*	$\rho = 0.924$. DNAm change over time −0.4% ± 4.7.	
		Decrease in DNAm over an 11–20 year interval	*INSIGF*	$\rho = 0.649$. DNAm change over time −0.9% ± 3.7.	
		Increase in DNAm over an 11–20 year interval	*KCNQIOT 1*	$\rho = 0.307$. DNAm change over time 1.0% ± 2.8.	
		Decrease in DNAm over an 11–20 year interval	*APOC1*	$\rho = 0.956$. DNAm change over time −0.1% ± 3.4.	
Cord and whole blood	Age	Genome-wide study finding significant ($p < 0.05$) change in DNAm in 159 CpGs in males and 149 CpGs in females; 98 CpGs were shared in males and females.	Genome-wide DNAm	Among the 98 sites changing significantly in both sexes, 2% changed from intermediate to hypomethylation, 27% changed from intermediate to hypermethylation, and 71% changed from hypermethylation to the intermediate category.	Wang et al. [86]

Foundational, direct evidence for the influence of epigenetic factors on mental illness over the life course was initially obtained through work conducted on rodent animal models. Ground-breaking work by Meaney and colleagues demonstrated that early-life experiences have an immediate yet lasting impact on epigenetic patterning of genes involved in the stress response: Rat pups born to mothers who provide a high degree of licking and grooming, as well as arched back nursing, show reduced levels of hippocampal DNA methylation and increased gene expression at the *NR3C1* glucocorticoid receptor locus—an important modulator of the stress response and integral component of the hypothalamic-pituitary-adrenal (HPA) axis—compared with pups born to mothers who provide less licking, grooming, and non-arched back nursing [26]. These differences emerge during the first week of life, persist until adulthood, and are accompanied by increased histone H3K9 acetylation and greater transcription factor binding in the high maternal care group [26]. Most importantly, these epigenetic changes were concordant with differences in HPA axis responses to stress during adulthood, whereby the offspring of the more "attentive" mothers showed a more modest increase in corticosterone in response to restraint stress compared with their peers born from less attentive mothers. This pattern of relative *NR3C1* hypermethylation has been reflected in results from human-based studies relating such variation to phenotypes reflective of depression (i.e., high cortisol): Increased maternal depressed/anxious mood, assessed prenatally during the third trimester, showed an association with increased DNA methylation at a predicted *NGFI-A* binding site in cord blood-derived DNA, and with increased salivary cortisol stress responses at 3 months postnatally [93].

Genes involved in the HPA axis have been a particular focus of study in life course epigenetic influences on psychiatric disorders, which to date (and perhaps by necessity) have been conducted based on retrospective case-control designs. Although, as described above, initial work focused on the gene encoding the glucocorticoid receptor (GR), more recent work has focused on *FKBP5*, in part due to its importance in modulating stress reactivity in local cellular contexts. FKBP5 is present in the complex containing the GR and modulates the ability of cortisol to bind to the GR; when cortisol binds to the GR complex, FKBP5 dissociates [94]. Activated GR then translocates into the nucleus [95,96], where it binds glucocorticoid response elements (GREs) and acts as a transcription factor, initiating transcription of *FKBP5* and recruiting other transcription factors. *FKBP5* is critically important in regulating the GR complex and is a negative regulator of GR activity. When FKBP5 is bound to the chaperone complex, the affinity with which cortisol binds the GR decreases, thus decreasing the amount of GR that translocates to the nucleus [94]. Over-induction of *FKBP5* following stress-related cortisol secretion has been linked to impaired negative feedback of cortisol [94] and prolonged cortisol release [97]. Numerous studies have implicated *FKBP5* polymorphisms in increasing risk for later psychiatric disorders, including MDD [98], PTSD [95], and suicide [99]. Such gene–environment interactions may well be mediated by epigenetic factors; a recent focused study of this locus showed that childhood maltreatment is associated with reduced *FKBP5* DNA methylation levels in GREs [100]. Individuals carrying the risky "A" allele of the SNP most strongly associated with lifetime risk of PTSD among those exposed to childhood maltreatment (i.e., rs1360780) also showed significantly reduced DNA methylation in a GRE located in intron 7 of the gene if they had been exposed to childhood maltreatment (CM), compared with the non-CM-exposed risk allele carriers and those with the protective "GG" genotype (whether exposed or unexposed to CM) [100]. Importantly, these SNP–DNA methylation associations were not observed when analyzed in relation to adult trauma, suggesting a developmentally sensitive window in which epigenetic factors may be "set" to levels that can predispose genetically vulnerable, CM-exposed individuals to later psychiatric disorders. Although these findings provide critical

insight into the mechanistic basis for how ELA translates into increased risk for psychiatric disorders later in life, it will be important to explore whether these results, obtained from a retrospective case-control design for the salient exposure (CM), are supported by future studies based on prospective collection of CM exposure, biological, and psychopathology data. Emerging work that assesses epigenetic variation in association with ELA [101,102] and other stressful exposures [103] more proximate to the occurrence of the actual stressor stands to make important contributions in this regard, provided that additional follow-up biological and psychopathological data are collected on these individuals.

Conclusions

As alluded to throughout this chapter, epigenetic epidemiology is in its relative infancy, and this is no less true for epigenetic studies of psychiatric disorders. Here we have attempted to provide a non-exhaustive overview of the field, summarizing the current state of the literature while acknowledging the particular challenges associated with psychiatric epigenetic epidemiology studies. For truly population-based research, most studies will continue to focus on DNA methylation, the most stable epigenetic mark that is tractable to study in a high-throughput format. Similarly, although microarray-based profiling may miss important regions of epigenetic variation, this will likely remain a preferred approach, given its genome-scale coverage and relative affordability. Current guidance regarding appropriate sample sizes for epigenetic epidemiology studies is limited: With respect to DNA methylation (but also true of other epigenetic measures), power will depend on the entire DNA methylation spectrum in cases and controls [88], which is currently unknown for most psychiatric and other complex disorders; recommendations about sample size will thus need to evolve with emerging data. Although there is no "one size fits all" approach to such studies, future work in the field is likely to benefit most if multiple measures of genomic variation (i.e., transcriptional and DNA sequence) are integrated into epigenetic studies of psychiatric disorders, in order to gain a more complete picture of the functional relevance of observed epigenetic variation [87]. Pairing these with longitudinal study designs would be especially informative, particularly if initial screens—perhaps on MZ twins discordant for the disease of interest—identifies epigenetic variation that could then be investigated for existence prior to disease onset [87,88]. In addition, analyses of existing data will likely need to be periodically reviewed, as more information is learned about the extent of intra- and inter-individual epigenetic variation over time across tissues and in disease and non-diseased states, so that disease-associated epigenetic variants can be more precisely identified. Genome-wide association studies of psychiatric disorders have established standards for establishing positive associations that include controlling for multiple hypothesis testing and replicating positive findings in independent cohorts; similar strategies may eventually be adopted by epigenome-wide association studies of psychiatric disorders once the distribution of population-level epigenetic variation is better understood.

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Glossary

Causal inference The process by which investigators determine whether a disease or outcome of interest is a consequence of an exposure that is hypothesized to produce the disease. A key consideration here is whether the putative cause occurs prior to the disease/outcome of interest.

Confounding Occurs when the effect of an exposure is "mixed together" with the effect of another variable, leading to a biased estimate of association with the outcome (i.e., disease) of interest. Statistical models can control for suspected confounding variables in order to arrive at an adjusted measure that focuses on the uniform (or close to uniform) effect that the exposure has on the outcome of interest.

Effect size A descriptive statistic that indicates the strength of a relationship between an exposure and outcome; often (but not always) expressed as the difference in mean outcomes of an exposed versus unexposed group divided by the standard deviation of the outcome in the unexposed group.

Longitudinal study A study that involves sampling participants at two or more time points, ideally before and after disease onset.

Retrospective case-control study A study that involves selecting participants from unrelated individuals with known diagnoses (i.e., cases), as well as a comparable set of controls, with both sets of participants drawn from the same source population.

Reverse causation Occurs when the exposure–disease association is actually a consequence (rather than a cause) of the disease itself; for example, in a sample of individuals with and without a given disease, any observed disease-associated epigenetic variation (i.e., the exposure) could be a result of having the disease itself.

CHAPTER

Laboratory Techniques in Psychiatric Epigenetics

7

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KEY CONCEPTS

- Laboratory techniques for analyzing DNA methylation and histone modifications are discussed.
- These techniques are applicable to studies ranging from single nucleotide and locus-specific measurements to global and genome-wide studies

ABBREVIATIONS

3C Chromosome conformation capture
5-fC 5-formylcytosine
5hmC 5′-Hydroxymethylcytosine
5mC 5′-Methylcytosine
5mdC 5-Methyl-2′-deoxycytidine
ac-H3K9K14 Acetylated histone H3 at lysines 9/14
CGRA Combined glycosylation restriction analysis
CHARM Comprehensive high-throughput array for relative methylation
ChIP Chromatin immunoprecipitation
CMS Cytosine-5-methylenesulfonate
COBRA Combined bisulfite restriction analysis
COREs Clusters of open regulatory elements
CpG Cytosine–phosphate–guanine
DMRs Differentially methylated regions
DNMT DNA methyltransferase
ELISA Enzyme-linked immunosorbent assay
ENCODE Encyclopedia of DNA Elements
ePCR Emulsion PCR
FAIRE-seq Formaldehyde-assisted isolation of regulatory elements
FRET Fluorescence resonance energy transfer
GLIB Glucosylation, periodate oxidation, biotinylation
GWAS Genome-wide association study
HDAC Histone deacetylase
HELP HpaII tiny fragment enrichment by ligation-mediated PCR
HPLC High-performance liquid chromatography
HRM High-resolution melt
LC-MS Liquid chromatography/mass spectroscopy
LinDA Linear DNA amplification
LINE Long interspersed nucleotide elements
MALDI-TOF Matrix-assisted laser desorption/ionization–time-of-flight
MBD Methyl-CpG-binding domain
MeCP2 Methyl-CpG-binding protein 2
MeDIP Methylated DNA immunoprecipitation
MIP Methylation-independent PCR
MIRA Methylated CpG island recovery assay
MLPA Multiplex ligation-dependent probe amplification
MNase Micrococcal nuclease
MS-HRM Methylation-sensitive high-resolution melting
MS-MLPA Methylation-specific multiplex ligation-dependent probe amplification
MSP Methylation-specific PCR
MS-qFRET Methylation-specific quantum dot fluorescence resonance energy transfer
Ms-SNuPE Methylation-sensitive single nucleotide primer extension
NDRs Nucleosome-depleted regions
NGS Next-generation sequencing
OxBS-seq Oxidative bisulfite sequencing
PCR Polymerase chain reaction
PFC Prefrontal cortex
PTSD Posttraumatic stress disorder
Q2ChIP Quick and quantitative ChIP
QD-FRET Quantum dot FRET
RNA-seq RNA sequencing
RRBS Reduced representation bisulfite sequencing
SMRT Single-molecule real-time
TAB-seq TET-assisted bisulfite sequencing
TET Ten–eleven translocation
WGBS Whole-genome bisulfite sequencing

Introduction

Epigenetics is defined as changes in gene expression or cellular phenotype, caused by mechanisms other than changes in the underlying DNA sequence. These changes may be induced in response to environmental or internal factors and include different components of epigenetic control, such as nucleic acid modification, histone modifications, or non-coding RNAs. Regarding nucleic acid modification, DNA methylation, mainly of cytosine nucleotide at the carbon 5 position (5mC), is a common epigenetic mark involved in epigenetic regulation of gene expression in many eukaryotes and is often found in the sequence context CpG (i.e., cytosine followed by a guanine). Promoter methylation is typically associated with repression. Recently, the Encyclopedia of DNA Elements (ENCODE) Project Consortium [1], using methods for genome-wide epigenetic analyses, reported that levels of DNA methylation correlated with chromatin accessibility in the genome. They also found that 96% of the 1.2 million CpGs measured exhibited differential methylation in at least one of 147 different cell types analyzed.

In recent years, we have been experiencing a revolution in the field of genetics. The development of new technologies such as next-generation sequencing (NGS) is allowing deep (i.e., high coverage) sequencing and the detection of various epigenetic modifications. In addition, new sensitive methods have been developed to analyze different conditions, types of samples, and classes of epigenetic marks. This chapter provides an overview of the various technologies that can be suitable, in neuroscience studies, for the quantitative global and locus-specific analysis of DNA methylation and chromatin. Shifting focus from "gene-centered" to "genome-wide" approaches, with developments in NGS technologies and biostatistical tools to analyze such gigabases of DNA data, promises to further enhance our understanding of genome architecture and its consequences on gene regulation and genome stability in the central nervous system (CNS) in both health and disease.

DNA methylation

It was in 1975 that Riggs and Holliday independently proposed that chemical modifications of DNA, such as methylation (i.e., the addition of a methyl group on certain cytosines), could influence gene expression. Following studies would provide further support, including that of McGhee and Ginder who, using restriction enzymes that distinguish between methylated and unmethylated DNA, demonstrated that the *beta-globin* locus was essentially unmethylated in cells that expressed beta-globin but methylated in other cell types that did not express this gene [2].

Since these early experiments, we now have multiple methods to study DNA methylation and quantify 5′-methylcytosine (5mC) levels either globally or locus specifically. This chapter will briefly cover the distinctions and attributes of the pretreatment methods for methylation screening techniques, including sodium bisulfite, enzyme restriction, and affinity purification. It will then discuss the merits of various analytical methods incorporating the pretreatments such as sodium bisulfite sequencing, methylation-specific PCR using restriction enzymes, and methylated DNA immunoprecipitation (MeDIP) (see Table 7.1). I will also explore newer techniques for the detection of 5′-hydroxymethylcytosine (5hmC), a derivative of 5mC, and the development of novel techniques that avoid the use of any of the aforementioned pretreatments, such as SMRT and Nanopore methods.

Table 7.1 Techniques for Studying DNA Methylation According to the DNA Pretreatments and Resolution of the Detection (Global, Gene-Specific, or Genome-Wide)

	Sodium Bisulfite Conversion	Enzyme Restriction	Affinity Enrichment
Global	Alu/LINE1-PCR, –pyrosequencing, or –HRM	HPLC, LC-MS	5mC ELISA
Gene-specific	MSP-PCR, MethyLight, MS-HRM, COBRA, single-clone sequencing, Ms-SNuPE,[a] *in situ*-MSP, MS-qFRET,[a] pyrosequencing, MassARRAY,[a] bisulfite patch PCR[a]	HpaII-PCR, MS-MLPA,[a] methyl-qPCR	MeDIP, MBD-ChIP, MIRA
Genome-wide, chip	Infinium, Golden Gate	HELP, CHARM	MeDIP-chip, MBD-chip, MIRA-chip
Genome-wide, NGS	RRBS, WGBS, bisulfite-seq	RRBS, methyl-seq, HELP-seq	MeDIP-seq, MIRA-seq
5hmC	OxBS-seq, TAB-seq, anti-CMS	PvuRts1I, CGRA	hMeDIP, GLIB, anti-CMS

[a]*Multiplex options.*

Global quantification of DNA methylation

Numerous antidepressants and mood stabilizers have been described to modulate epigenetic patterns through histone deacetylase (HDAC) and/or DNA methyltransferase (DNMT) inhibition, leading to global and widespread genome changes in DNA methylation [3]. Considering that these epigenetic mechanisms participate in fundamental developmental and regulatory processes, increasing attention has been focused on the epigenetic changes resulting from prescribed drugs. In addition, there is high interest in examining global 5mC levels in association with diet and lifestyle. For example, it would be important to quantify global patterns of DNA methylation in order to evaluate the effect of certain diets and food stuffs, such as folic acid, for example, in prenatal life, childhood, or adulthood [4]. A growing list of environmental factors and pollutants such as polycyclic aromatic hydrocarbons are also being realized to have global effects on DNA methylation [5].

Several methods can be used to detect the total, overall (i.e., global) levels of 5mC in a genome. DNA can be digested into single nucleotides and total genomic 5mC can be quantified by high-performance liquid chromatography (HPLC) or liquid chromatography/mass spectroscopy (LC-MS). Although the methods may be relatively laborious, they can provide highly quantitative and reproducible results. For example, Zhang and colleagues [6] reported detection limits of 0.5×10^{-15} mol for 5-methyl-2′-deoxycytidine (5mdC) using DNA inputs between 1 ng and 1 µg. They were able to use this technique to assess global DNA methylation in fetal tissue from anencephalus and spina bifida cases revealing reductions in brain global DNA methylation compared with control samples, whereas no differences were found in skin tissue, suggesting a possible role of hypomethylation in neural tube defects.

Simpler methods have been developed for rapid detection and quantification of global 5mC, such as a competitive enzyme immunoassay or enzyme-linked immunosorbent assay (methDNA-ELISA) that can measure 2–6% methylation in mammalian genomes with 10 ng of input DNA [7]. Such assays

have been used to measure levels of global DNA hypomethylation in leukocytes with increasing age, for example [8]. It is also possible to use immunohistochemistry to investigate global 5mC and 5hmC. This can be used on postmortem brain samples, for example, to give a level of region and cell specificity. Chouliaras and colleagues [9] used this to demonstrate decreases in the hippocampal levels of 5mC and 5hmC in patients with Alzheimer's disease. Furthermore, levels of 5mC and 5hmC showed a significant negative correlation with amyloid plaque load in the hippocampus, demonstrating the further usefulness of this method for colocalization experiments.

The vast majority of global methylation identified is assumed to be localized to interspersed repetitive elements [10]. Several polymerase chain reaction (PCR)-based methods, such as high-resolution melt (HRM) curve analysis, real-time PCR, or pyrosequencing-based technologies, have been developed that assess methylation at repetitive DNA elements such as Alu and long interspersed nucleotide elements (LINE) as a function of global methylation. For example, the methylation status of repetitive elements (i.e., Alu, LINE-1 and SAT-α) using quantitative PCR pyrosequencing revealed increased LINE-1 methylation in lymphocytes from patients with Alzheimer's, compared with healthy volunteers, that correlated further with poorer performances in mini-mental state examinations [11].

Higher resolution analyses of DNA methylation

As global methylation analyses provide no information on the genomic positions at which nucleotide methylation is altered, other high-resolution approaches are needed. Most approaches to measure locus or site-specific 5mC are based on one of three main pretreatments: sodium bisulfite, restriction enzyme digestion, or affinity capture.

Sodium bisulfite treatment

During PCR amplification, DNA methylation information is lost as DNA polymerase cannot distinguish between methylated and unmethylated cytosines. Sodium bisulfite treatment provides a way to allow DNA to be modified prior to PCR, thus enabling the methylation information to be preserved. This chemical deaminates cytosine to uracil—importantly, the rate of deamination of 5mC to thymine is much slower than the conversion of cytosine to uracil, and therefore one assumes that the only cytosines remaining after treatment are those that were methylated. Thus, during subsequent PCRs, uracil residues are replicated as thymine residues, and 5mC residues are replicated as cytosines (Figure 7.1). The classic sodium bisulfite treatment protocol of Frommer and colleagues from 1992 [12] remains one of the most widely used techniques and is the backbone of a variety of commercial kits (e.g., QIAGEN, Zymo Research, Invitrogen, Millipore) for this purpose. Patterson et al. [13] provided excellent instruction for a non-kit-based protocol; however, though considered by many as the gold standard for determining DNA methylation as it allows single-CpG resolution, one should be aware that this treatment is not without its disadvantages. Although the conversion rate of unmethylated cytosines tends to be very high (around 99% when performed under appropriate conditions) [14], small subsets of DNA copies may show far lower conversion rates [15]. This can be influenced by sequence; for example, some promoter regions may be more prone to incomplete conversion, and can also depend on DNA quality [15]. In addition, sodium bisulfite-based methods generally require relatively larger quantities of sample DNA, as this heavily degrades under sodium bisulfite treatment. Finally, sodium bisulfite conversion

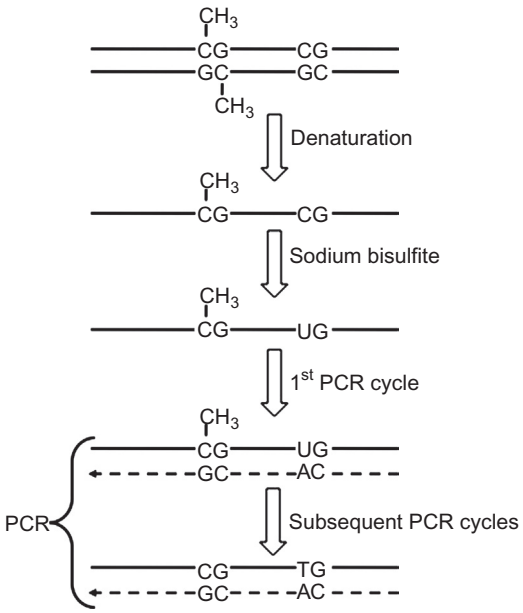

FIGURE 7.1

Bisulfite conversion. DNA is denatured and then treated with sodium bisulfite to convert unmethylated cytosine (C) bases to uracil (U). This is then converted to thymine (T) by PCR.

cannot discriminate between 5mC and 5hmC. Nevertheless, sodium bisulfite conversion is arguably the most conventional, widely employed pretreatment approach.

Restriction enzyme digestion

Some restriction enzymes are 5mC sensitive; for example, HpaI, HpaII, and MspI will not cut sequences in which a cytosine is methylated. Using corresponding 5mC-insensitive restriction enzymes, which have identical recognition sequences (isoschizomers) though cut indifferently of methylation, it is possible to distinguish methylated from unmethylated cytosines. There are actually over 100 different methylation-sensitive restriction enzymes and isoschizomers available (Table 7.2); their applicability is defined by the size and number of cleavage fragments they produce and their presence within recognition sequences. These can be used in conjunction with PCR amplification (e.g., HpaII–PCR) or Southern blot analysis. The EpiTect Methyl qPCR System (SABiosciences, QIAGEN), using methylation-sensitive enzymes to digest DNA followed by quantitative real-time PCR, allows customers to purchase plates of different disease- or pathway-focused gene panels as a screening tool. A number of other related systems are available from other commercial suppliers.

There are several methods combining methylation-sensitive restriction enzyme digestion with high-throughput DNA analyses such as microarrays or NGS. Comprehensive high-throughput arrays for relative methylation (CHARM) uses McrBC (a restriction enzyme that recognizes two closely spaced

Table 7.2 Recognition Sequences and Cleavage Sites of Methylation-Sensitive Restriction Enzymes

Restriction Enzyme	Recognition Site	Restriction Enzyme	Recognition Site
AatII	GACGT/C	HaeII	RGCGC/Y
AciI	C/CGC	HgaI	GACGC (5/10)
AclI	AA/CGTT	HhaI	GCG/C
AfeI	AGC/GCT	HinP1I	G/CGC
AgeI	A/CCGGT	HpaII	C/CGG
AscI	GG/CGCGCC	Hpy99I	CGWCG/
AsiSI	GCGAT/CGC	HpyCH4IV	A/CGT
AvaI	C/YCGRG	KasI	G/GCGCC
BceAI	ACGGC (12/14)	MluI	A/CGCGT
BmgBI	CAC/GTC	NaeI	GCC/GGC
BsaAI	YAC/GTR	NarI	GG/CGCC
BsaHI	GR/CGYC	NgoMIV	G/CCGGC
BsiEI	CGRY/CG	NotI	GC/GGCCGC
BsiWI	C/GTACG	NruI	TCG/CGA
BsmBI	CGTCTC (1/5)	PaeR7I	C/TCGAG
BspDI	AT/CGAT	PmlI	CAC/GTG
BspEI	T/CCGGA	PvuI	CGAT/CG
BsrBI	CCGCTC (-3/-3)	RsrII	CG/GWCCG
BsrFI	R/CCGGY	SacII	CCGC/GG
BssHII	G/CGCGC	SalI	G/TCGAC
BstBI	TT/CGAA	SfoI	GGC/GCC
BstUI	CG/CG	SgrAI	CR/CCGGYG
ClaI	AT/CGAT	SmaI	CCC/GGG
EagI	C/GGCCG	SnaBI	TAC/GTA
FauI	CCCGC (4/6)	TliI	C/TCGAG
FseI	GGCCGG/CC	XhoI	C/TCGAG
FspI	TGC/GCA		

Note: In cases where the cleavage site is some distance away from the recognition site, numbers are given in parentheses indicating the distance between the last given nucleotide and the cleavage site. W = A or T; Y = C or T; R = G or A.

methylated cytosines) to digest genomic DNA at CpG-rich regions that is then detected for CpG methylated CpG using a microarray [16]. Another similar assay, HpaII tiny fragment enrichment by ligation-mediated PCR (HELP), as the name suggests uses the methylation-sensitive restriction enzyme HpaII to digested DNA fragments before hybridizing to microarray [17].

A new method, described later, known as reduced representation bisulfite sequencing (RRBS), uses these enzymes to enrich CpG islands for methylation analysis [18]. One new class of restriction enzymes (MspJI homologs FspEI, LpnPI, AspBHI, RlaI, and SgrTI), rather than being methylation sensitive or insensitive, are actually dependent on methylation for restriction. This property has recently been demonstrated to allow an alternative method to examine the methylation state of a genome [19].

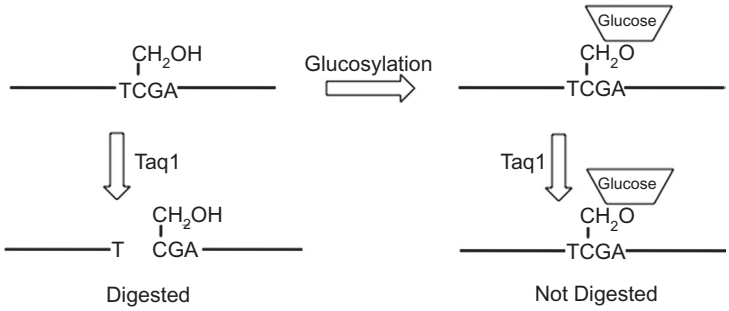

FIGURE 7.2

Combined glycosylation restriction analysis (CGRA) for 5hmC detection. This method uses a methylation-insensitive restriction enzyme (e.g., TaqαI) coupled with selective glucose labeling of 5hmC.

Furthermore, there is renewed interest in using restriction enzyme-based methods in identification, detection, and quantification of 5hmC from 5mC [20]. PvuRts1I, for example, exhibits specificity toward 5hmC over 5mC. The combined glycosylation restriction analysis (CGRA) method uses T4 phage β-glucosyltransferase to add a glucose moiety to 5hmC followed by digestion with restriction enzymes such as TaqαI or MspI, which are sensitive to glucosylated 5hmC [21] (Figure 7.2).

Affinity capture

The most commonly reported DNA affinity capture method is methylated DNA immunoprecipitation (Me-DIP), which uses a 5mC-specific antibody, or methyl-CpG binding domain (MBD) protein. Briefly, genomic DNA is fragmented to an average size of around 400 bp (using either sonification or restriction enzymes), bound to 5mC antibody or MBD, combined with Sepharose® or magnetic beads, and then purified from uncaptured DNA. Gene fragments can then be quantified using real-time PCR to test for methylation status at a single locus or the pulled-down DNA may be used for microarray analyses (MeDIP-chip, MBD-chip, MIRA-chip) to test numerous positions across the genome. Newer methods, known as MeDIP-seq, are combining this with next-generation sequencing technologies and using immunoprecipitation to enrich for the portion of the genome containing either 5mC or 5hmC, depending on the antibody used, followed by high-throughput sequencing to provide total genome-wide coverage. MeDIP-seq was used to generate the first whole-genome methylation profile of a mammalian genome [22] and has since been successfully used to provide methylation profiles of several tissues including brain. For example, a genome-wide study of promoter methylation by Labonte and colleagues [23] using MeDIP in conjunction with a promoter-tiling array (Agilent Technologies) identified 362 promoters that differed in methylation in hippocampi between individuals with severe abuse during childhood compared with controls.

The different combination of pretreatment methods and subsequent molecular biology techniques, such as gene-specific analyses, DNA microarrays, and high-throughput sequencing (all described below), generates an abundance of techniques for mapping DNA methylation in the most suitable way from a gene to a genome-wide scale.

Gene-specific quantification of DNA methylation

Most gene-specific methylation analysis methods are primarily PCR based, with the vast majority using DNA pretreated with sodium bisulfite as the template. Two different strategies have been used in the design of primers for such reactions. The first approach is based on the design of primers that specifically amplify methylated or unmethylated templates and is adopted by methylation-specific PCR (MSP) and quantitative MSP. The second approach is based on primers that amplify a region of the desired template including CpG islands, no matter what the methylation status is. In this case, methylation-independent PCR (MIP) is first performed and then information on the methylation status of that region is obtained through post-PCR analyses techniques such as bisulfite sequencing, restriction digestion, single-strand conformation analysis, and high-resolution melting.

MethPrimer [24] is a program for designing PCR primers for MSP and sequencing-sequencing or restriction-restriction PCR. The freely available online software also allows prediction of CpG islands in DNA sequences. There are also several online programs for CpG island identification such as CpG plot (EMBL-EBI), which screens for CpG islands meeting specific criteria in submitted DNA sequences. MethPrimerDB is a public database holding predesigned PCR primers for popular DNA methylation analysis methods [24].

Methylation-specific PCR methods

Methylation-specific PCR-based methods use specific primers for methylated versus unmethylated DNA in a PCR on bisulfite-treated DNA. An unmethylated-specific primer will only amplify sodium bisulfite-converted unmethylated DNA, whereas a methylated primer is specific for sodium bisulfite-treated methylated DNA [25]. This can be combined with real-time PCR (quantitative-MSP) using SYBR® Green, fluorescently labeled MSP primers, or TaqMan® probes [26] to allow sensitive quantification of DNA methylation. This can be further extended to a fluorescence-based technique called MethyLight, which uses forward and reverse primers with an inter-positioned probe to increase the degree of specificity, sensitivity, and flexibility [27]. These methods provide a relatively sensitive, fast, and cost-effective test for the methylation status of CpG dinucleotides, making the technique applicable to high-throughput analysis. A possible drawback of this procedure is the occurrence of false-positive results caused by the amplification of unconverted bisulfite DNA. To combat this, a nested two-stage MSP (nested MSP) is possible using primers that amplify only sodium bisulfite-modified DNA (but not discerning between methylated and unmethylated alleles) in the first PCR, which is then subjected to a second PCR using unmethylated and methylated-specific MSP primers [28].

Methylation-specific PCR can be coupled with *in situ* hybridization (*in situ* MSP) to enhance detection at the cellular level [29]. Two sections on slides are treated with sodium bisulfite and subjected to PCR with either unmethylated- or methylated-specific primers, followed by *in situ* hybridization with a digoxin-labeled probe. Although seemingly attractive, this method has yet to be well described in brain and neuronal studies.

Methylation-specific quantum dot fluorescence resonance energy transfer (MS-qFRET) combines the high specificity of MSP and the high sensitivity and simplicity of quantum dot FRET (QD-FRET) technology. Modified-modified DNA is subjected to PCR amplification with primers that would differentiate between methylated and unmethylated DNA. Quantum dots are then used to capture PCR amplicons and determine the methylation status via fluorescence resonance energy transfer (FRET).

Advantages include its low intrinsic background noise, high resolution, and high sensitivity, allowing detection of as little as 15 pg of methylated DNA in the presence of a 10,000-fold excess of unmethylated alleles. It requires relatively little input. For example, it has been applied to sputum samples that contain very low concentrations of DNA, and it can be adapted to multiplexing reactions and high-throughput screening [30], thus offering great potential for use in clinical studies.

Bisulfite sequencing approaches

The gold standard in DNA methylation analysis has traditionally been the sequencing of bisulfite-modified and PCR-amplified DNA [12] using MIP primers. PCR products can be sequenced, using dideoxy sequencing, directly or as single clones. Sequencing of cloned PCR products provides information on individual molecules, whereas direct sequencing provides an estimate of the average methylation status of each CpG site in all of the molecules. In clone reading, the ratio of methylated to unmethylated molecules in a sample can be determined if enough clones are sequenced. This method was used, for example, to detect DNA methylation changes at the arginine vasopressin (AVP) enhancer in the hypothalamus in response to early-life stress in mice [31]; however, this method is relatively time-consuming. There are also some reports that sequencing of single clones can sometimes be affected by a cloning bias, depending on the nature of the sequence [32]. To combat this, it is possible to combine bisulfite sequencing with digital PCR. Here, a DNA sample is analyzed in distributive fashion over multiple reaction chambers, allowing for the absolute quantification of discrete template DNA molecules, as well as sequestration of non-specific primer annealing templates into negative chambers, thereby increasing the signal-to-noise ratio in positive chambers [33]. A further approach is to perform bisulfite patch PCR involving highly multiplexed bisulfite PCR and sequencing across many samples. Using this method, Varley and Mitra [34] were able to amplify 94 targeted gene promoters simultaneously in the same reaction. By incorporating sample-specific DNA barcodes into the amplicons, they could analyze 48 samples in a single run of the 454 Life Sciences (Roche) FLX sequencer.

An alternative approach to traditional dideoxy (Sanger) sequencing is pyrosequencing. This is based on the detection of light emitted during synthesis of the complementary strand by an exonuclease-deficient DNA polymerase. When nucleotides are incorporated, pyrophosphate is released and converted to adenosine triphosphate (ATP) by the enzyme ATP sulfurylase. The ATP molecules provide energy for the enzyme luciferase to oxidize luciferin in a reaction that generates light. The four different nucleotides are added sequentially to enable base calling. Adapted to methylation analysis with bisulfite-modified DNA, this method yields quantitative information on single CpG sites [35]. Some drawbacks are that accuracy decreases with the distance of the CpG from the forward primer, which limits the length of products that can be analyzed in a single sequencing reaction [36], while the long stretches of thymine often found in bisulfite-modified DNA can affect reproducibility.

Combined bisulfite restriction analysis (COBRA) is a technique that allows sensitive quantification of DNA methylation levels at a specific genomic locus on a DNA sequence in a small sample of genomic DNA [37]. A variation of bisulfite sequencing, this method combines bisulfite conversion-based polymerase chain reaction with restriction digestion (Figure 7.3). Originally developed to reliably handle minute amounts of genomic DNA from microdissected paraffin-embedded tissue samples, this is still widely used. Recently, Sheikh and colleagues [38] used this method to establish DNA methylation patterns at the *dipeptidyl peptidase 6* gene during retinoic acid-induced neuronal

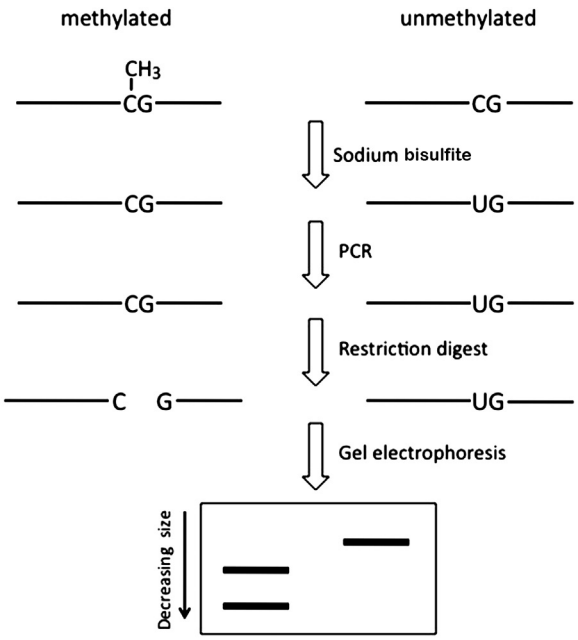

FIGURE 7.3

Analysis of methylation states using sodium bisulfite-treated DNA and restriction enzyme. DNA is treated with sodium bisulfite and then digested by a restriction enzyme. Unmethylated CpG dinucleotides are converted by bisulfite treatment, and the resulting PCR product is digested by a restriction enzyme specific for the sequence.

differentiation of P19 cells. Though the method is less labor intensive than sequencing of cloned PCR products and more cost effective, the main limitation is that many CpG sites cannot be analyzed because the restriction enzymes that would be appropriate for recognizing the sequence context are not available.

The base composition of PCR products derived from sodium bisulfite-modified templates is methylation dependent; hence, methylated and unmethylated PCR products show different melting profiles when subjected to thermal denaturation. The methylation-sensitive high-resolution melting (MS-HRM) protocol, as described by Wojdacz and Dobrovic [39], is based on the comparison of the melting profiles of PCR products from unknown samples with profiles specific for PCR products derived from methylated and unmethylated control DNAs. The protocol consists of PCR amplification of bisulfite-modified DNA with primers designed to proportionally amplify both methylated and unmethylated templates and subsequent high-resolution melting analysis of the PCR product. An interesting example of the use of MS-HRM and the genotype-epigenotype interaction was reported by Perroud et al. [40], who were able to use this to measure the percentage of methylation at regulatory regions of the *BDNF* gene in the lymphocytes of subjects with borderline personality disorder linking higher numbers of childhood trauma with higher methylation status.

Methylation-sensitive single nucleotide primer extension (Ms-SNuPE) is a technique that can be used for rapid quantification of methylation at individual CpG sites [41]. Treatment of genomic DNA with sodium bisulfite is used to convert unmethylated cytosine to uracil while leaving 5mC unaltered. Strand-specific PCR is performed to generate a DNA template for quantitative methylation analysis using Ms-SNuPE. SNuPE is then performed with oligonucleotides designed to hybridize immediately upstream of the CpG sites being interrogated. Reaction products are electrophoresed on polyacrylamide gels for visualization and quantification by phosphorimage analysis. This method can be used for high-throughput methylation analysis and rapid quantification of cytosine methylation suitable for a wide range of biological investigations. Multilocus Ms-SNuPE assays have recently been established for the simultaneous quantification of methylation at multiple methylation imprinted loci for diagnostic testing for different congenital imprinting disorders such as Silver–Russell syndrome, Beckwith–Wiedemann syndrome, and transient neonatal diabetes in parallel, as well as for multilocus methylation defects [42]. A further modification of the protocol using a methylated CpG island recovery assay (MIRA), to specifically detect 5mC but not 5hmC, in combination with SNuPE allows analysis of allele-specific CpG methylation at differentially methylated regions (DMRs) of imprinted genes. Lee et al. [43], for example, were able to use this assay to reveal that placenta exhibits less DNA methylation bias at several DMRs compared to yolk sac, amnion, brain, heart, kidney, liver, and muscle. A microarray-based version of this method was recently introduced as a semi-quantitative high-throughput method [44].

Methylation-specific multiplex ligation-dependent probe amplification (MS-MLPA) is a variant of the multiplex ligation-dependent probe amplification (MLPA) technique, in which copy number detection is combined with the use of a methylation-sensitive restriction enzyme. Methylation-specific MLPA compares one undigested sample for copy number detection and one digested sample for methylation detection, with MS-MLPA probes for target sequences containing the methylation-sensitive restriction site. This is another technique that has been widely used for the detection of epigenetic imprinting diseases. Park and colleagues [45], when comparing MS-MLPA with MSP in quantifying the methylation status of the O^6-methylguanine DNA methyltransferase (MGMT) promoter in glioblastoma samples, found the diagnostic accuracy of MS-MLPA method to be better than that obtained with MSP.

DNA analysis by matrix-assisted laser desorption/ionization–time-of-flight (MALDI-TOF) mass spectrometry provides a highly sensitive method of detection based on differences in fragment sizes that have been cleaved according to the methylation status of multiple CpG sites. Briefly, bisulfite-treated DNA is tagged during PCR with the T7-promoter sequence and *in vitro* transcription is performed using T7R, DNA polymerase, and a specific nucleotide, after which the RNA transcripts are cleaved at specific bases with RNase A [46]. The resulting fragmentation patterns are analyzed using MALDI-TOF MS (Figure 7.4). The introduction or removal of cleavage sites by C-to-U conversions, or shift in fragment mass by G-to-A conversions in the amplified reverse strand, allows one to determine the specific pattern of DNA methylation by analyzing the fragment sizes of the resulting fragments. This approach (marketed by Sequenom, Inc. as the EpiTYPER® MassARRAY®) allows one to quantify methylation levels of several CpG sites within a 200- to 600-bp region and has been used for numerous promoter methylation studies, such as those on *NR3C1* and *MAOA* promoters in lymphocytes of subjects exposed to childhood adversity, for example [47]. Recent applications are allowing multiplexing (iPLEX) for numerous regions or multiple single CpG sites (up to 30 or more) at different genomic regions simultaneously in a reaction.

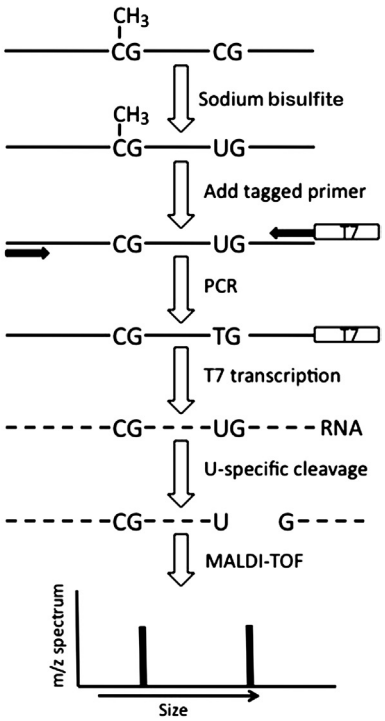

FIGURE 7.4

MALDI-TOF mass spectrometry analysis of CpG methylation. Genomic DNA is modified by sodium bisulfite to convert unmethylated cytosines to uracils followed by PCR amplification with a forward primer and reverse primer carrying the T7 promoter sequence. RNA transcription generates G sites at originally methylated C sites, and U- or G-specific cleavage with RNase is performed. RNA fragments are then subjected to MALDI-TOF analysis. By comparison with the profile of *m/z* values of all fragments in the samples, the methylation status of genes can be found.

Methylated DNA affinity enrichment approaches

Methylated DNA immunoprecipitation (MeDIP) and methylated CpG island recovery assays (MIRAs) use either a monoclonal antibody to 5mC (or 5hmC) (i.e., MeDIP) or a methylated DNA-binding protein (i.e., MBD-ChIP) immunoprecipitate fragmented DNA (Figure 7.5). The resulting enrichment of methylated DNA in the immunoprecipitated fraction can be determined by PCR-based methods to assess the methylation state of specific regions. Sweatt and colleagues [48] used MeDIP to demonstrate that infant maltreatment resulted in methylation of *BDNF* regulatory regions through the lifespan to adulthood that dovetailed reduced gene expression in the adult prefrontal cortex (PFC). Further, MeDIP can be combined with large-scale analyses using microarrays or deep sequencing (as described below). Davies and colleagues [49], for example, have been able to present annotated maps of the brain methylome using MeDIP combined with ultra-deep sequencing (MeDIP-seq) to profile the methylomic landscape across multiple dissected brain regions and blood obtained from multiple individuals [49].

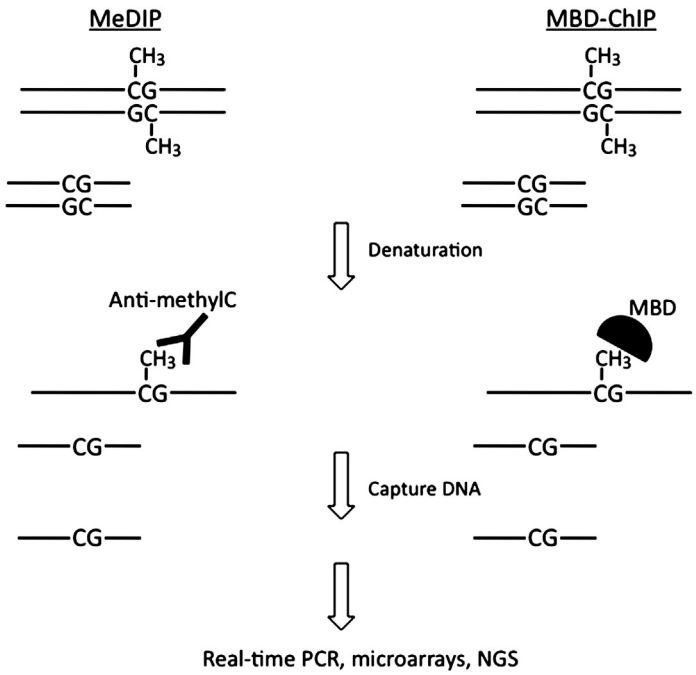

FIGURE 7.5

MeDIP and MBD-ChIP analysis of methylation. Genomic DNA is denatured and then affinity purified with either an antibody (MeDIP) or a methyl-binding protein (MBD-ChIP) that can be attached to a column, agarose beads, or magnetic beads.

Genome-wide DNA methylation analysis

Rapidly developing technologies are increasing the capacity to assess multiple locus-specific DNA methylation sites throughout a genome in combination with microarrays or NGS approaches. These hold the promise of providing increasingly greater density genome-wide DNA methylation studies.

Microarray-based methylation approaches

The main classes of microarray-based methods developed to quantify DNA methylation are those using sodium bisulfite, methylation-sensitive restriction enzymes, or methylated DNA enrichment. Early microarray studies of DNA methylation utilized spotted arrays made by individual laboratories or microarray facilities; however, advances in technology have seen the development of high-quality commercial oligonucleotide arrays, such as bead arrays made by Illumina®, lithographic arrays made by Affymetrix, adaptive lithographic arrays made by NimbleGen, and inkjet arrays manufactured by Agilent. Both the design and the technology behind an array influence their utility in methylation analysis. For example, the Illumina arrays are designed to analyze converted-converted DNA, while

other arrays are well suited for restriction enzyme- and affinity-based assays. Bisulfite-based microarrays are designed using oligonucleotide hybridization probes targeting the CpG sites of interest. With Infinium methylation assays (Illumina), researchers can quantitatively interrogate methylation sites at single nucleotide resolution, profiling up to 12 samples in parallel to deliver high-throughput power while minimizing the cost per sample. The HumanMethylation27 BeadChip™ lets researchers interrogate 27,578 CpG loci, covering more than 14,000 genes, while the 450K Infinium Methylation BeadChip covers over 450,000 methylation sites, making it ideal for screening genome-wide association study (GWAS) populations. Sample requirements for the Illumina Infinium are between 0.5 µg and 1 µg bisulfite-treated human DNA. Recently, Mehta and colleagues [50] used the 450K array to examine the impact of different early environments on DNA methylation in peripheral blood cells from patients with posttraumatic stress disorder (PTSD).

Coupling MeDIP with DNA microarrays (MeDIP-chip) allows one to obtain relative methylation levels at multiple loci represented by those probes included on an array. This is expressed relative to the input DNA or to another sample. MeDIP-seq was used to generate the first whole-genome methylation profile of a mammalian genome [22] and has since been successfully used to provide methylation profiles of several tissues including peripheral blood cells [51] and was recently used to conducted genome-wide analysis to identify DMRs in the brain between humans and rhesus macaques [52]. Agilent DNA methylation microarrays are designed to interrogate known CpG islands, promoters of human and mouse genomes, or defined regions such as those found in ENCODE. These arrays are specifically designed for analysis of methylated DNA derived from affinity-based isolation methods such as MeDIP and requires 5-µg genomic DNA. Roche NimbleGen also provides affinity-based methods for enrichment of methylated DNA fragments (i.e., MeDIP or MBD methods) and requires 1.5-µg input DNA. Importantly, methylation changes identified by microarray experiments will continue to require validation by one or more of the locus-specific methods and, although methylation microarrays are powerful tools, an important limitation is that they analyze only a small part of the CpG sites of the genome.

Next-generation sequencing-based methylation analysis

Following completion of the Human Genome Project, the high demand for low-cost sequencing has given rise to a number of high-throughput NGS technologies. These new sequencing platforms allow high-throughput sequencing for a wide range of applications, such as whole-genome sequencing as *de novo* or resequencing, targeted resequencing, transcriptome profiling, and gene regulation studies. NGS technology has also been adapted to epigenomic research, including DNA methylation and chromatin (described later) profiling at high resolution and relatively low cost. Sodium bisulfite conversion followed by NGS (Bisulfite-seq or MethylC-seq) has become an increasingly used method for performing epigenetic profiles of mammalian genomes [53]. Whole-genome bisulfite sequencing provides coverage at a single-base pair resolution and is the most comprehensive technique, covering more than 90% of cytosines in the human genome. Recently, Lister et al. [54] used MethylC-seq to reveal extensive global DNA methylation reconfiguration during mammalian brain development. This allowed the researchers to discover high levels of cell-type-specific intragenic methylation patterns and to identify over 100,000 developmentally dynamic and cell-type-specific differentially CG-methylated regions enriched at putative regulatory regions of the genome. Importantly, it also enabled the detection of highly conserved non-CG methylation (mCH) in neurons. Several sequencing strategies and platforms are available, and the choice of platform and methodology is dependent on the scientific application

with regard to throughput, read-length, accuracy, cost per run, cost per megabase, initial costs, size, and technology. One must also consider the capacity for extensive bioinformatics analysis.

Roche/454 sequencing of converted-converted DNA

Roche/454 FLX technology is based on pyrosequencing. After the incorporation of each nucleotide by DNA polymerase, a pyrophosphate is released which further takes part in downstream light-producing reactions; the amount of light is proportional to the incorporated number of nucleotides. Briefly, DNA is fragmented, and adapters are ligated to both ends and mixed with agarose beads carrying complementary adaptor sequences (i.e., each bead is associated with a unique DNA fragment). These are isolated in individual micelles and emulsion PCR (ePCR) amplified to generate millions of copies of the single fragment on the surface of the bead. Each bead is placed in a well of picotiter plate, enzymes are added for the pyrosequencing, and nucleotides are flowed over in a predetermined sequence while a camera records the light emitted from each bead. The major disadvantage of this method is the misinterpretation of homopolymers (consecutive nucleotides, such as AAA or CCC). However, bisulfite patch PCR can be used to enable highly multiplexed bisulfite PCR and sequencing across many samples. Varley and Mitra [34] were able to use this method to amplify 94 targeted gene promoters simultaneously in the same reaction. Incorporating sample-specific DNA barcodes into the amplicons allowed them to examine 48 samples in a single run of the 454 Life Sciences (Roche) FLX sequencer. The method requires small amounts of starting DNA (250 ng) and does not require a shotgun library construction. The method was highly specific: 90% of sequencing reads aligned to targeted loci. This method also further allows detection of single nucleotide polymorphisms to test whether DNA methylation accumulates on specific alleles.

SOLiD™ sequencing of converted-converted DNA

Similarly to the Roche 454, in SOLiD™ (Applied Biosystems®) sequencing, DNA is fragmented and adapters ligated to the ends. These are attached to small paramagnetic beads and subjected to emulsion PCR amplification. Rather than the use of DNA polymerases, as for the Illumina and Roche 454, sequencing by synthesis is performed by utilizing DNA ligase. Each cycle of sequencing involves the ligation of a degenerate population of fluorescently labeled universal octamer primers. SOLiD has also been adapted for genome-wide DNA methylation analysis. SOLiD technology differs from other high-throughput deep sequencing approaches, as it detects two nucleotides at a time by ligation and predicts one of the four colors associated with those specific two bases. Bormann et al. [55] assessed the capabilities of the SOLiD approach for large-scale bisulfite sequencing and generated two libraries (in-solution converted-converted and gel converted-converted library) from *Escherichia coli* DH10B. These libraries were then amplified on magnetic beads by ePCR using the standard SOLiD protocol, with the exception that additional deoxyadenosine triphosphate (dATP) and deoxythymidine triphosphate (dTTP) were added to the aqueous ePCR phase to compensate for the low complexity of the converted-converted libraries. Interestingly, this study demonstrated the advantage of bisulfite color versus base space sequencing, a finding that could assist in the analysis of more complex mammalian genomes, such as humans. The sequencer adopts the technology of two-base sequencing based on ligation sequencing. On a SOLiD flow cell, the libraries can be sequenced by eight base-probe ligation which contains ligation site (the first base), cleavage site (the fifth base), and four different fluorescent dyes (linked to the last base). The fluorescent signal is recorded during the probes complementary to the template strand

and vanished by the cleavage of the probes' last three bases. Also, the sequence of the fragment can be deduced after five rounds of sequencing using ladder primer sets.

Illumina sequencing of converted-converted DNA

Again, DNA is fragmented and adapters ligated onto the ends. One of the adapters is then hybridized on a flow cell and ePCR-amplified locally around the hybridized DNA to generate clusters that will have the exact same molecule. A primer is added to anneal to the adaptor and a mixture of modified nucleotides, each carrying a base-specific fluorescent label, is added. The flow cell is placed under a microscope and fluoresces, as light is emitted when bases are incorporated on each one of those clusters (a blocked 3′-OH group ensures the incorporation of only one nucleotide at a time).

Ion torrent

As above, DNA is fragmented, adaptors are ligated, and a single DNA template is affixed to a bead and clonally amplified using ePCR. The beads are then loaded onto the chip, where, on average, each well contains a single bead. Deoxynucleotide triphosphates (dNTPs) are flowed over the surface of the bead in a predetermined sequence. A single proton is released for every nucleotide incorporated during the flow, resulting in a pH change in the surrounding solution which is detected by an ionic sensor.

Genome approaches

Gene-specific information on DNA methylation patterns can be acquired by sequencing bisulfite-converted DNA for a single genomic locus. As previously described, NGS can be modified to allow multiplexing of PCR fragments and samples. For genome-wide analysis, many modified versions of the standard bisulfite sequencing have been developed, as whole-genome Bisulfite-seq requires extensive bioinformatic analysis in addition to expense.

One widely employed modification is reduced representation bisulfite sequencing (RRBS), which Meissner et al. [56] developed in order to reduce the amount of nucleotides needed to be sequenced to 1% of the genome. This involves using restriction fragment size selection to select a portion of the genome enriched for CpG islands and gene regulatory sequences allowing coverage of approximately 12% of all CpG dinucleotides and 84% of all CpG islands in the human genome including the majority of promoters. By combining RRBS and Illumina sequencing, the authors were able to generate DNA methylation maps comparing mouse pluripotent stem cells with differentiated cells covering most of the CpGs in the mouse genome, along with a representative sampling of conserved non-coding elements, transposons, and other genomic features. The results of this study demonstrated that DNA methylation patterns are dynamic and undergo global changes when pluripotent stem cells differentiate. Agilent, in a related approach, offers a SureSelect Methyl-Seq kit for specific capture of potentially methylated regions, such as promoters and CpG islands.

The MeDIP-seq method first uses an antibody to pull down the methylated cytosines and then amplifies the methylated cytosines. The MBD-seq technique is the same, except it uses a methylation-binding domain protein for the pull down. These methods are less expensive than whole-genome bisulfite sequencing and target genome-wide CpGs more efficiently than the reduced representation

method. These methods, therefore, could be useful if cost is an issue and researchers want to target more than just CpG islands. A recent report comparing the targeted approach of the Infinium HumanMethylation450 BeadChip with the immunoprecipitation and sequencing-based method, MeDIP-seq (using an Illumina sequencer), for the detection of DMRs revealed that both methods show a good positive correlation on a genome-wide scale and can both be used successfully to determine differentially methylated loci in RefSeq genes, CpG islands, shores, and shelves. MeDIP-seq however, allowed wider interrogation of methylated regions of the human genome, including thousands of non-RefSeq genes and repetitive elements, and the detection of nearly twice as many DMRs as the array-based method [57].

With the costs of sequencing declining, high-resolution, genome-wide measurement of DNA methylation is now possible using whole-genome bisulfite sequencing (WGBS), a process whereby input DNA is treated with sodium bisulfite and sequenced. This allows profiling of DNA methylation across the entire genome and is the only approach that provides a complete picture of the entire DNA methylome at single nucleotide resolution. An application of WGBS by Lister et al. [58], named MethylC-seq, was able to compare DNA methylation profiles of an embryonic stem cell line and a fibroblast cell line at about 30× coverage using the Illumina GA II.

5hmC approaches

As early as 1970, researchers identified the epigenetic mark 5hmC in the brains and livers of rats, as well as in the brains of both frogs and mice [59]. Two recent studies rediscovered this mark in mouse embryonic stem cells and Purkinje neurons [60,61], identifying enzymes that can modify 5mC—namely TET1, which is able to actively promote DNA demethylation. Numerous studies support the importance of a global balance between 5mC and 5hmC in the genome as being critical for regulating gene expression in maintaining cellular functions. A recent study discovered that neuronal TET1 regulates DNA methylation and expression levels of several neuronal memory-associated genes controlling contextual fear memory [62], strengthening the hypothesis that 5hmC might be involved in gene expression regulation.

To be able to identify exactly which genomic sites are modified by 5hmC requires the development of further methods, as bisulfite sequencing is unable to specifically detect 5hmC, which behaves like its precursor, 5mC, in bisulfite conversion experiments. In fact, some bisulfite sequencing data may need to be revisited to verify whether the detected modified base is 5mC or 5hmC. As previously described, MeDIP may be used with an antibody to 5hmC to enrich for this modification. However, these methods are unable to detect 5hmC at a single-base resolution. The other previously mentioned restriction enzyme methods for 5hmC can only detect cytosine residues in specific sequences (e.g. TaqαI at TCGA). Further methods are needed to enable high-resolution 5hmC mapping. Oxidative bisulfite sequencing (OxBS-seq) is based on the selective chemical oxidation of 5hmC to 5-formylcytosine (5-fC), which enables bisulfite conversion of 5fC to uracil (Figure 7.5). This has been applied to embryonic stem cell genomic DNA, revealing high levels of 5hmC in CpG islands associated with transcriptional regulators and in long interspersed nuclear elements, suggesting that these regions might undergo epigenetic reprogramming. Another method, TET-assisted bisulfite sequencing (TAB-seq), when combined with traditional bisulfite sequencing, can be used for mapping 5hmC at base resolution and quantifying the relative abundance of 5hmC as well as 5mC (Figure 7.6). Lister and colleagues

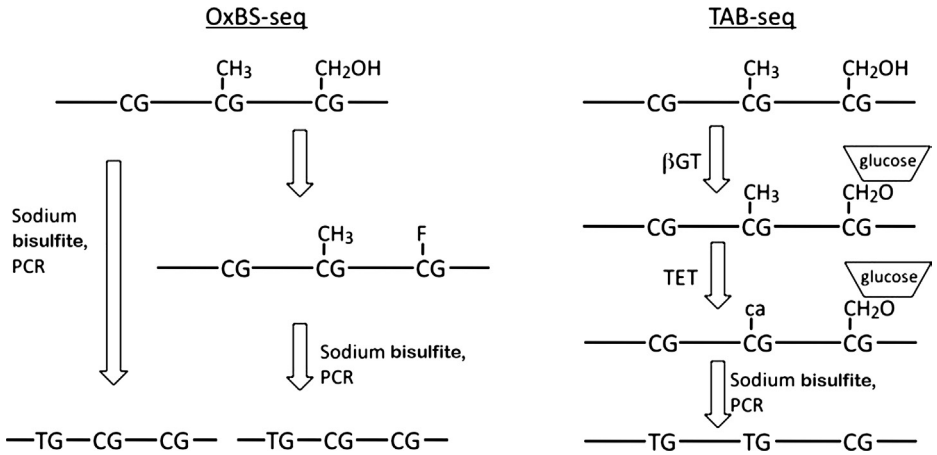

FIGURE 7.6

OxBS-seq and TAB-seq for 5hmC detection. OxBS-seq involves oxidation of 5hmC to 5fC by KRuO4 which is subsequently converted into T by bisulfite treatment and PCR. This is compared with bisulfite sequencing without $KRuO_4$ treatment in which the 5hmC remains as a C. In TAB-seq, 5hmC is protected from TET-mediated oxidation and bisulfite conversion by βGT-catalyzed glucosylation. 5mC is then oxidized by TET to 5caC and subsequently converted into T after bisulfite treatment and PCR.

[58], in their genome-wide study on mammalian brain development, used this technique to detect 5hmC at single-base resolution, revealing that this mark is present in fetal brain cells at locations that lose CG methylation and become activated during development. Other promising new methods include GLIB (glucosylation, periodate oxidation, biotinylation) [63] and the anti-CMS protocol (bisulfite conversion of 5hmC to cytosine-5-methylenesulfonate [CMS] followed by immunoprecipitation using an antibody against CMS) [64]. The global quantification of 5hmC is becoming crucial to understanding the dynamic epigenetic mechanisms and changes that occur in the cells.

The aforementioned NGS technologies, while providing huge amounts of information encoded within nucleic acid sequences, are not without their limitations: short read lengths, amplification biases, reliance on sodium bisulfite (which has limitations in itself), and relatively large amounts of starting material. Recently, Pacific Biosciences developed single-molecule real-time (SMRT) sequencing, which is capable of detecting DNA methylation without the need for bisulfite conversion and is amenable to long read lengths, possibly enabling mapping of DNA methylation patterns in even highly repetitive genomic regions. In SMRT sequencing, DNA polymerase catalyzes the incorporation of fluorescently labeled nucleotides into complementary nucleic acid strands. SMRT sequencing utilizes arrival times and duration of the resulting fluorescence pulse to generate information about polymerase kinetics, which allows direct detection of modified nucleotides in the DNA template. SMRT sequencing was able to detect and differentiate N6-methylcytosine, 5mC, and 5hmC [65].

Another method potentially negating the use of DNA pretreatments for DNA methylation analyses are nanopores, which use the principle of ionic current spectroscopy to electrically interrogate individual DNA molecules with the sensitivity to discern subtle structural motifs (Figure 7.7). Fabrication of these devices typically involves the physical sputtering of a single nanometer-sized aperture in a

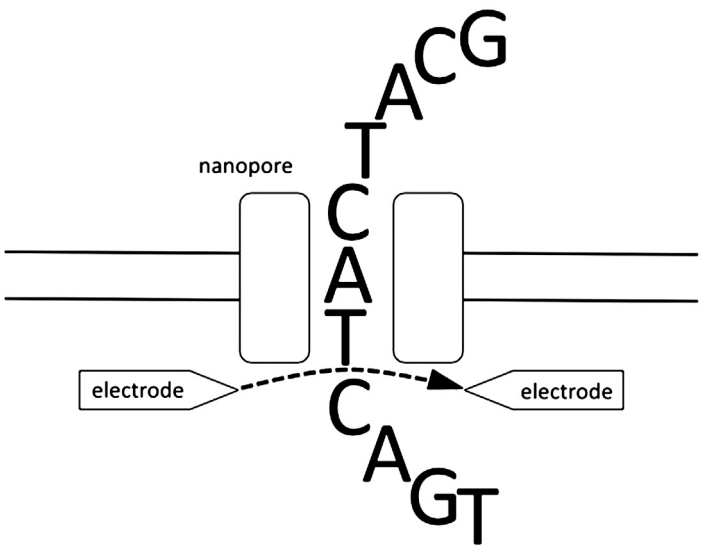

FIGURE 7.7

Nanopore DNA sequencing. As a DNA strand ratchets through a nanopore, the different bases interfere with the voltage across the pore to different degrees, allowing the sequence to be read.

dielectric membrane using a focused electron beam. The electrophoretic transport of biomolecules through these nanoscale pores has allowed the study of various biophysical phenomena at the single-molecule level, with potential applications in DNA sequencing and medical diagnostics. Recently, methylated and unmethylated DNA has been examined optically in nanofluidic channels using fluorescently labeled proteins bound to the methylation sites. Nanopore-based ionic current spectroscopy, however, is ideal for single-molecule epigenetic analysis, as it eliminates the need for optical measurements. Using nanopore-based ionic current spectroscopy, the differentiation of 5mC from C has previously been demonstrated by passing these individual nucleotides through a biological nanopore, requiring an exonuclease-based cleaving of the bases from the original molecule. Although to date ionic current measurements obtained using a solid-state nanopore have yet to differentiate methylated from unmethylated single molecules of DNA [66], further developments in this field may yet yield a highly efficient and sensitive method for DNA methylation analysis.

Chromatin

Chromatin consists of a complex of DNA and core histone proteins, which act as a spool to package DNA to fit inside the nucleus. These core histone proteins (H2A, H2B, H3, and H4) are subject to posttranslational modifications. The most common modifications are acetylation, phosphorylation, and methylation, all of which are capable of influencing the rate of gene transcription by controlling physical access to the gene. Both phosphorylation and acetylation are associated with transcriptional activation, while the transcriptional consequences of histone methylation are dependent on the number and location of the methyl

groups. In recent years, the field of neuroscience has begun to realize the importance of these epigenetic mechanisms in both development and cognition. A critical role for chromatin modifications in learning has now been demonstrated in several rodent behavioral models, including tasks dependent on the hippocampus and cortex. Moreover, chromatin modifications play an important role in psychiatric disorders such as schizophrenia, depression, and drug addiction.

Global histone analyses

Western blot analysis on nuclear extracts with antibodies raised against specific histone modifications is a relatively simple technique able to reveal the type, tissue location, and degree of histone posttranslational modifications produced by an experimental manipulation. For example, measuring global histone acetylation levels in the rat hippocampus revealed differential acetylation of H3, H4, and H2B at early stages of spatial or fear memory formation: H3K9K14 acetylation was mostly responsive to any experimental conditions compared with naive animals, whereas H2B N-terminus and H4K12 acetylations were mostly associated with memory for either spatial or fear learning [67]. Other options are to use ELISA. Rao and colleagues [68], for example, found increases in global histone H3 acetylation in the frontal cortex of bipolar disorder cases. Indeed, numerous studies have linked global histone changes to several psychiatric diseases, including neurodegenerative and neurodevelopmental disorders. Such global histone studies may also be important analytical tools for exposures to drugs or toxins known to interfere with histone modifications. For example, nickel is known to interfere with histone modifications in cell culture studies; a study on lymphocytes of workers at a nickel refinery in Jinchang, China, revealed global elevations in H3K4me3 and deceases in H3K9me2 [69].

Sequence-specific and genome-wide histone analyses

Chromatin immunoprecipitation (ChIP)

Recently, incredible progress has been seen in characterizing histone modifications on genome-wide scales, since the first development of chromatin immunoprecipitation (ChIP) by John Lis and David Gilmour in 1984. Using ultraviolet irradiation to covalently cross-link proteins in contact with neighboring DNA, followed by immunoprecipitation with an antibody to bacterial RNA polymerase, they were able to map RNA polymerase to different regions of genes in intact bacterial cells. A year later they used the same methodology to study the distribution of eukaryotic RNA polymerase II on fruit fly heat shock genes [70]. Two months later, Mark Solomon and Alexander Varshavsky developed the idea to use formaldehyde as a cross-linking reagent to examine the gene locations of histone H4 [71].

The ChIP technique has developed into a powerful and widely applied tool to analyze the spatial and temporal association of specific proteins (e.g., modified histones, DNA-binding proteins, transcription factors) within native cells *in vivo*. It further forms the basis of numerous related adapted approaches. The basic principle of this method is that living cells are treated with formaldehyde to cross-link proteins and DNA within ~2 Å of each other. As a result, proteins are covalently bound to their target sequences on the DNA and provide a snapshot of transcription factor occupancy. Next, the cross-linked DNA is fragmented into pieces of 300–600 bp and protein–DNA complexes are immunoprecipitated by antibodies against the protein of interest (Figure 7.8). This allows enrichment for specific DNA

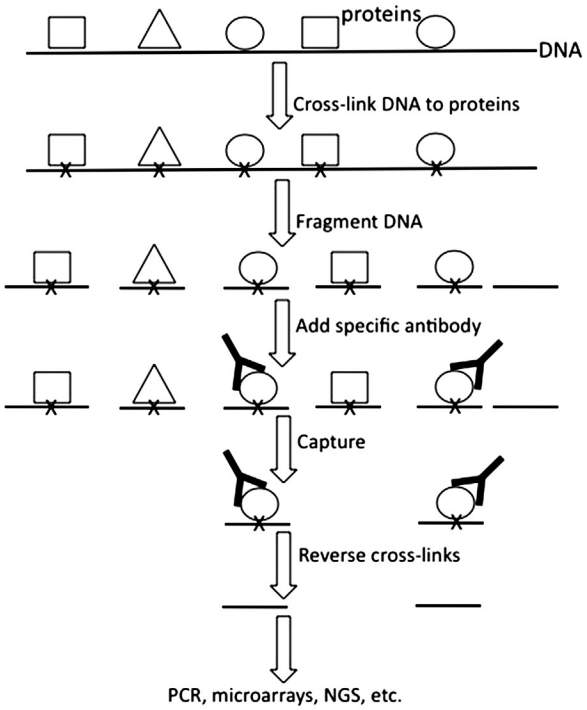

FIGURE 7.8

Chromatin immunoprecipitation (ChIP) procedure. Cells are initially treated with a cross-linking agent (e.g., formaldehyde) that covalently links DNA-interacting proteins to the DNA. The genomic DNA is then isolated and sheared (e.g., sonication) into a suitable fragment size distribution (usually around 300 bp). An antibody that specifically recognizes the protein of interest is then added and immunoprecipitation is used to isolate appropriate protein–DNA complexes. The cross-links are then reversed, and the DNA fragments are purified and measured.

sequences, which can then be identified through amplification by PCR [72], hybridization to microarrays (ChIP-on chip) [73], cloning [74], or NGS (ChIP-seq) [75].

Neurons of related functions typically cluster in nuclei that are generally small in size (often limited to hundreds to thousands of cells, especially in rodents) and rather heterogeneous. A major factor to consider for ChIP on brain tissue is possibly the limited numbers of cells available and the fact that the recovery rate of cross-linked DNA varies from 1 to 10% of total DNA in the starting material. One major step forward was a reduction of the time scale (from 3 days to within 1 day) in the "Fast ChIP" protocol [76] by employing an ultrasonic water bath to reduce the antibody incubation step and Chelex® resin to elute the immunoprecipitated DNA fragments from the protein A beads, which can then be directly used as PCR templates without further purification. However, this method requires relatively large cell samples ($0.5-1 \times 10^6$) due to relatively low DNA yield and quality [76]. Another method omitted the DNA clean-up step by performing PCR directly on the immunoprecipitated cross-linked

DNA [77]; however, PCR detection was unreliable and frequently failed in case of small tissue samples [78]. Moreover, it has been reported that this ChIP technique is not compatible with qPCR analysis dependent on the nature of the carrier beads used, as some of them, such as magnetic beads, appear to interfere with quantification of the SYBR Green signal during real-time PCR [79]. Combining high throughput with shortened immunoprecipitation and wash steps, the EpiQuick™ ChIP kit (Epigentek™) is based on a microplate format whereby all steps are performed without sample transfers, offering some potential for automation [80]. However, the kit still relies on a minimum input of 500,000 cells per reaction. A quick and quantitative ChIP (Q2ChIP) assay addressed the issue of limited starting material by allowing chromatin preparation from relatively large cell numbers, which could then be diluted and aliquoted, allowing many samples to be prepared in parallel, and stored for use in numerous independent chromatin immunoprecipitations [81]. To scale down further the number of cells required for the starting material, the microChIP (or μChIP) assay was developed in which as few as 100 cells or small ($1\,mm^3$) tissue microdissection samples can be used per ChIP sample. In addition, it can be completed within 1 day, depending on the length of the immunoprecipitation time required [81]. To allow multiple ChIPs to be performed simultaneously, a 96-well microplate-based ChIP (Matrix ChIP) assay offers the potential for automation [80].

Another *in vivo* ChiP protocol has been customized further by Murgatroyd and colleagues [79] for use on brain microdissection samples and to facilitate the handling of multiple samples at the same time. Separate cell and nuclear membrane lyses are performed to increase the relative concentration of chromatin in the sample. A sonicating water bath is used to achieve an even distribution of the energy (the energy of sonication in traditional sonicators is dependent on the depth of the sonication probe in the liquid) and so increasing reproducibility and the possibility to process many samples in parallel. The Bioruptor® sonicator (Diagenode) has been extensively validated for this purpose. Immunoprecipitations are performed overnight in a ChIP dilution buffer to increase maximum binding, followed by incubation with magnetic Dynabeads™ protein G or A and washing with increasingly stringent buffers. The elution of DNA from the magnetic beads, reversal of cross-linking, and proteinase K digestion are performed in one step followed by protein denaturation at 95°C. The DNA is then further purified using spin columns. A series of samples can be simultaneously processed in a highly reproducible manner by this cost-effective method in a reasonably short time frame [78].

Two further protocols have been recently developed to address the problem of analyzing the very low quantity of DNA extracted after performing a ChIP, by using post-ChIP DNA amplification. Nano-ChIP-seq [82] uses custom primers that form a hairpin structure at the 5′ end to prevent self-annealing when being added and allow PCR amplification. The primers also contain a BciVI restriction site that allows the direct addition of Illumina sequencing adapters for DNA library preparation. The second protocol uses T7 RNA polymerase linear DNA amplification (LinDA), which overcomes amplification bias in the starting material, particularly in relation to GC content, which is generally problematic for PCR-based approaches. This has been successfully applied for transcription factor ERα using 5000 cells and for the histone modification H3K4me3 using 10,000 cells [83].

The above ChIP protocols use sonication to fragment chromatin resulting in libraries containing DNA molecules that are approximately 300 bases long, even though a protein typically binds only 6–20 bases. A further major factor in ChIP is background noise from DNA not bound by the target factor, which is why most ChIP experiments include an input control. Using lambda (λ) exonuclease to digest DNA immediately adjacent to formaldehyde cross-linked protein-bound DNA fragments (fixation is a barrier to the DNA digestion), ChIP-exo allows identification binding sites with single-base-pair

precision [84]. In addition, the exonuclease largely eliminates contaminating DNA, giving a 40-fold increase in the signal-to-noise ratio. Finally, it goes without saying that the success of a ChIP experiment depends to a huge degree on the development and validation of a highly specific antibody to the bound protein or modification.

Analyzing multiple binding events

DNA regulatory elements generally interact with an assortment of transcription factors in conjunction with various cofactors and histone modifications in order to regulate gene transcription. Therefore, it is attractive to be able to determine the cohabitation of several proteins in a particular developmental stage and cell type. Importantly, multiple experiments indicating different proteins or modifications at the same genomic location do not necessarily indicate that these are simultaneously binding or present in the same cell. Sequential ChIP (also known as re-ChIP) allows determining the *in vivo* colocalization of proteins interacting at a DNA sequence on the basis of double and independent rounds of immunoprecipitation [85]. McGill and colleagues [86], for example, were able to use this technique to demonstrate that methyl-CpG-binding protein 2 (MeCP2) preferentially associates with a transcriptionally inactive chromatin histone mark at the CRH promoter [86]. Two recently published assays allow linking DNA methylation to ChIP by subjecting ChIP-enriched DNA to bisulfite sequencing. Known as BisChIP-seq [87] and ChIP-BS-seq [88], these methods use similar steps but in a different order. One genome-wide experiment, for example, revealed that H3K27me3-modified histones can occur simultaneously with DNA methylation [87]. Such techniques allow for testing the association or influence of various chromatin marks and DNA binding factors on DNA methylation under various conditions. The previous ChIP methods only enable testing for those specific proteins or histone modifications chosen by the researcher, dependent on the antibody. Very recent strategies are establishing an integrative global proteomic profiling approach, or reverse ChIP involving a combination of a DNA probe and mass spectrometric analysis, to allow the unbiased identification of chromatin-associated proteins [89].

Gene-specific ChIP

Real-time PCR can be used to individually test numerous (depending on DNA quantity) specific gene regions in ChIP-enriched DNA. For example, Tang and colleagues [90] used ChIP-qPCR to measure levels of acetylated histone H3 at lysines 9/14 (ac-H3K9K14), two epigenetic marks associated with transcriptionally active chromatin, at the promoter regions of eight schizophrenia-related genes in 82 postmortem PFC samples from normal subjects and those with schizophrenia and bipolar disorder. SABioscience (QIAGEN) offers the purchase of panels of 96 primer assays (EpiTect ChIP PCR Array) for pathway- or disease-focused genes in 96-well plates with SYBR Green to which ChIP-enriched DNA is added. A further recent method for obtaining higher throughput is through the use of such technologies as NanoString® that allow the analysis of up to 800 target sequences (custom designed) in a single multiplexed reaction on as little as 10-ng ChIP-enriched DNA [91].

Genome-wide ChIP

A major driving force in genomic mapping of chromatin and DNA-binding proteins has been the development and improvement of high-throughput sequencing by combining ChIP with DNA

microarray (ChIP-on-chip) or sequencing (ChIP-seq) analysis techniques. ChIP-on-chip involves hybridizing the immunoprecipitated DNA fragments to either promoter or genome tiling DNA microarrays. Promoter tiling arrays, containing probes generally specific to gene promoters, tend to be of lower cost though may not cover all relevant regions, depending on the experimental focus. In genome tiling arrays, however, probes are included to cover entire genomes, allowing genome-wide analysis. ChIP-seq combines ChIP with NGS technologies (previously described), resulting in the production of millions of sequence reads during each run. Compared with ChIP-chip, ChIP-seq can be considered as being more quantitative (increasing the number of sequencing improves sensitivity), with lower bias due to the lack of any issues associated with cross-hybridization on arrays. Because the data obtained are sequence reads, ChIP-seq further offers the potential to detect mutations in binding-site sequences or to identify polymorphic sites associated with functional variation; that is, it is possible to investigate differences in the distribution of sequences containing one allele versus the other, indicating allelic effects on chromatin or DNA protein binding.

These ChIP-based approaches have enabled mapping of the epigenome in brain tissues, providing us with maps of H3K4me3 in human prefrontal cortex [92], for example, among numerous other marks and brain regions that are freely available for examination on the University of California Santa Cruz (UCSC) Genome Browser website. Both of these methods were also behind ENCODE, the decade-long multicenter study [1]. The consortium was able to map 11 histone modifications (H3K27ac, H3K27me3, H3K36me3, H3K4me1, H3K4me2, H3K4me3, H3K79me2, H3K9ac, H3K9me1, H3K9me3, H4K2) across numerous cells and tissues, including human neuronal cell lines, neuroblastomas, and glioblastomas, as well as mouse cerebellum, cortex, and cerebrum [93], in addition to providing transcriptome and cistrome (set of *cis*-acting targets of a *trans*-acting factor on a genome scale) data. ChIP, however, can only provide binding/modification information for a single epitope, whereas multidimensional analyses often require information about the coordinate binding of several factors and/or corresponding epigenetic modification of targets sites. To this aim, sequential ChIP assays (reChIP) can in principle be combined with massive parallel sequencing (reChIP-seq), although such methods are still under development [83]. Such ChIP-seq technologies pave the way to identifying chromatin modifications across a genome that might be changed in psychiatric disorders, induced by stress or antidepressant medications in specific brain regions in animal models or postmortem human brain. When further combined with data from RNA sequencing (RNA-seq) this could allow even deeper insight into the interplay between chromatin activity and gene-specific transcription.

Open chromatin

Several studies have shown that open chromatin regions, which often correspond to nucleosome-depleted regions (NDRs), associate with all known classes of active DNA regulatory elements, including promoters, enhancers, silencers, insulators, and locus control regions. A few assays have been developed to detect open chromatin and thus identify regulatory sites across the genome (Figure 7.9). FAIRE-seq (formaldehyde-assisted isolation of regulatory elements) [94] enriches nucleosome-depleted DNA using formaldehyde fixation and phenol–chloroform extraction, while DNase-seq employs the DNaseI enzyme to preferentially digest nucleosome-depleted sites, also known as DNaseI hypersensitive (HS) sites [95]. Another endonuclease-based method allows the determination of nucleosome occupancy and positioning by generating cuts using micrococcal nuclease (MNase) that digests preferentially within linker DNA between nucleosomes and in nucleosome-depleted regions. Song et al. [96]

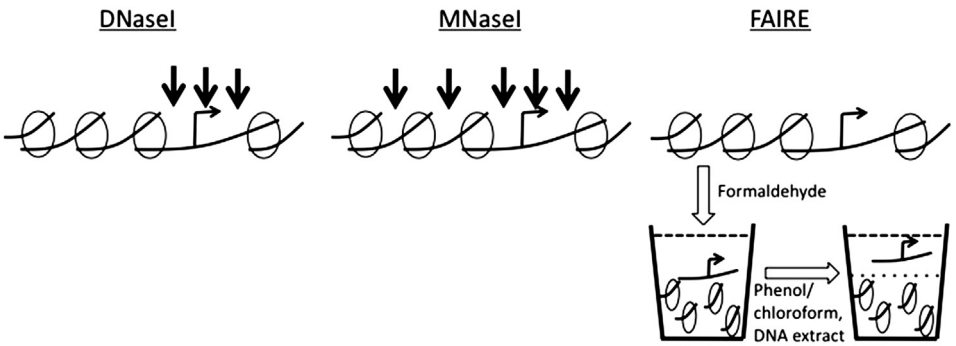

FIGURE 7.9

DNaseI, MNase, and FAIRE analyses of nucleosomes. DNaseI digests DNA free of DNA–protein interactions, such as nucleosome packaging. Micrococcal nuclease (MNase) digestion generates cuts preferentially within linker DNA between nucleosomes and in nucleosome-depleted regions. Formaldehyde-assisted isolation of regulatory elements (FAIRE) entails formaldehyde fixation of chromatin and subsequent separation of protein-free DNA by phenol–chloroform extraction. The resulting soluble fraction is enriched for nucleosome-depleted DNA, including sites of DNaseI hypersensitivity.

have combined DNase-seq and FAIRE-seq to generate genome-wide open chromatin maps spanning seven diverse human cell types. They identified clusters of open regulatory elements (COREs), typically located away from transcription start sites and containing DNA motifs recognized by regulators of cell-type identity. It is also possible to analyze FAIRE without NGS. Novaira and colleagues [97] were able to use this technique with real-time PCR to demonstrate a time-course response of kisspeptins inducing nucleosome depletion and the opening of chromatin within a kisspeptin response element (KsRE) of the GnRH promoter in the GT1-7 rat hypothalamic cell line.

A new variation of the previous "footprinting"-style methods has been recently developed; NOMe-seq uses a GpC methyltransferase (M.CviPI) that methylates all cytosines within the 5′-GC dinucleotide recognition sequence [98]. Next-generation sequencing then allows generating a high-resolution footprint of nucleosome positioning genome-wide while retaining DNA methylation information from the same DNA strand, at the single-cell level. The authors were able to use this method to demonstrate a striking anticorrelation between nucleosome occupancy and DNA methylation at CTCF regions that is not present at promoters.

Mapping chromatin interactions

Genomes are organized into three-dimensional structures, often adopting complex higher-order conformations that allow interactions between gene promoters and regulatory elements. Indeed, the expression of genes can be controlled by regulatory elements that are located at large genomic distances from their target genes, or even on different chromosomes. Regulatory elements can act at large genomic distances by engaging in direct physical interactions with their target genes, resulting in the formation of chromatin "loops." Thus, genes and their regulatory elements come within close spatial proximity irrespective of their relative genomic positions. Such long-range interactions can be

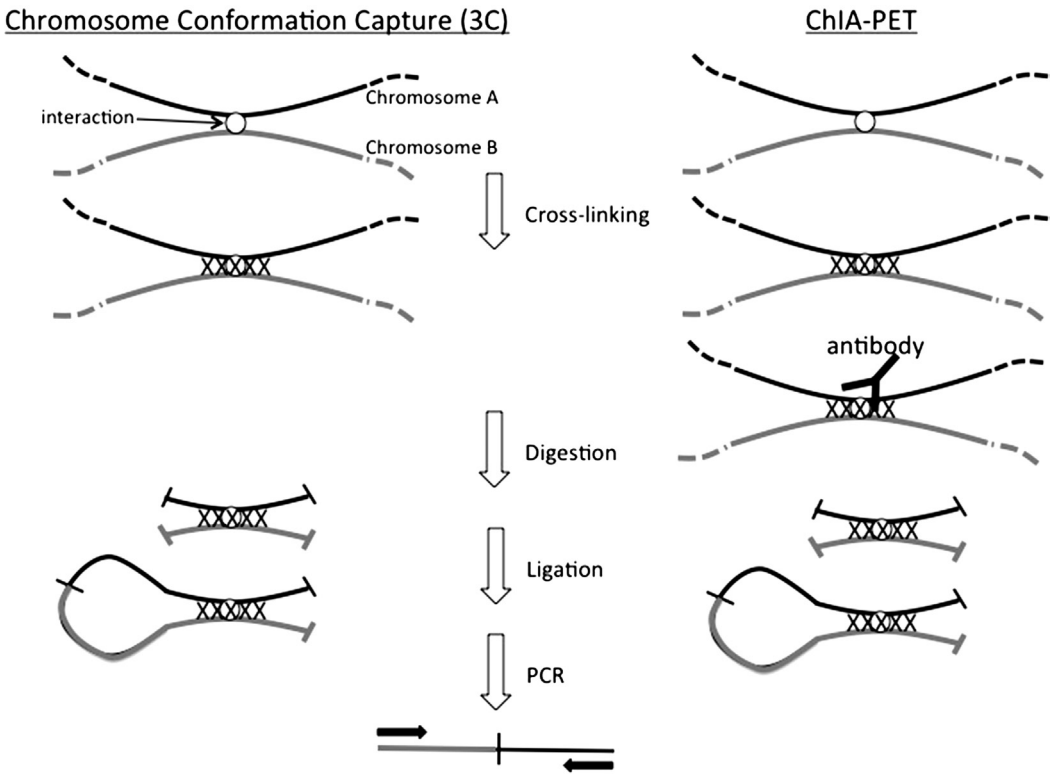

FIGURE 7.10

Chromosome conformation capture (3C) assay. Chromatin is cross-linked, digested with a restriction enzyme, and then ligated. Specific ligation products can be detected by PCR.

studied at high resolution using chromosome conformation capture (3C) technology (Figure 7.10), which utilizes formaldehyde cross-linking to trap physical interactions between loci located throughout the genome. Cross-linked cells are solubilized and chromatin is digested with a restriction enzyme. Chromatin is subsequently ligated under conditions that favor intramolecular ligation. After reversal of the cross-links, the DNA is purified and interaction frequencies between specific chromosomal loci are determined by quantifying the amounts of corresponding ligation products using PCR designed for regions of interest [99]. There is a variety of C-technologies (3C, 4C, 5C, Hi-C) that differ in their manner of detection and scope of what interactions they can probe: 3C allows testing interactions between only two known sites in the genome, 4C allows probing unknown binding elements of a known "bait" sequence, 5C identifies all regions of interaction within a given genome domain, and Hi-C probes all occurring interactions in an unbiased fashion genome-wide. Additional variants (ChIA-PET, ChIP-Loop) incorporate a protein precipitation step, thus allowing identification of genome interactions that involve a specific protein of interest [100]. Mitchell et al. [101] have very recently demonstrated the applicability of this chromosome conformation capture

technology to postmortem brain tissue, which allowed them to compare the tissue specificity of higher-order chromatin structures at the chromosome 6p22.2-22.1 schizophrenia and bipolar disorder susceptibility locus in PFC compared with other tissues and cells.

Conclusion

A large number of strategies have been used to examine epigenetic marks. Both DNA methylation and ChIP methods have progressed from small-scale candidate gene analysis to the ability to construct whole-genome methylation profiles. Clearly, no one method is best suited for every application. Researchers should conduct a preliminary analysis focused on the type of validation and information provided by each technique in order to select the best method fitting for their research. Furthermore, it has recently become apparent that, in addition to DNA methylation, there are other modifications of DNA, such as hydroxymethylation and non-CpG methylation, that play a role in epigenetic regulation of gene expression. These findings suggest that DNA modifications are more dynamic than previously thought. The importance of chromatin and DNA-binding proteins has motivated the continued development of experimental and analytical methods to better identify and characterize these interactions. Although ChIP-seq remains the standard for identifying binding site locations for individual proteins and histone modifications, the practical limitations of antibody development—a single factor/modification limit per experiment, the lack of functional annotation, and a static snapshot of a dynamic cell—require the use of complementary methods or extensions of ChIP-seq to provide a more complete picture of biological processes in the cell, especially transcriptional regulation. In the next few years, given the speed with which NGS has been adopted in epigenetic research and the ever-developing newer technologies, epigenetic methods will be effective tools for testing important gene–environment interactions in neuronal functioning.

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Glossary

5-hydroxymethylcytosine (5hmc) An epigenetic modification of DNA that can result from the oxidation of 5-methylcytosine by the TET family of enzymes.

5-methylcytosine (5mC) An epigenetic modification of DNA that usually occurs at CpG dinucleotides and often correlates with repressed gene expression.

Bisulfite conversion The deamination of non-methylated cytosine bases to uracil by treatment with sodium bisulfite ($NaHSO_3$) while methylated cytosines are resistant to bisulfite conversion.

Bisulfite sequencing Determining the sequence of bisulfite-converted DNA.

ChIP-on-chip Combining ChIP and DNA hybridization for genomic microarrays.

ChIP-seq Combining ChIP and NGS to analyze specific DNA–protein interactions genome-wide.

Chromatin The complex of DNA, histones, RNA, and other proteins that comprise the structural basis of chromosomes.

Chromatin immunoprecipitation (ChIP) A method used to identify proteins bound to DNA and the sequence to which they bind using an antibody to specifically immunoprecipitate the protein of interest while co-precipitating the bound DNA.

Chromosome conformation capture (3C) Allows mapping of local chromosome interactions through increased frequency of intramolecular ligation between fragments in close proximity.

Combined bisulfite restriction analysis (COBRA) DNA is subjected to bisulfite conversion and digestion with restriction endonucleases that are specific for sequences containing CpG sites.

Comprehensive high-throughput arrays for relative methylation (CHARM) Uses McrBC (a restriction enzyme that recognizes two closely spaced methylated cytosines) to digest genomic DNA and detect hypermethylated CpG sites using a microarray.

CpG islands Regions of DNA enriched for CG dinucleotides. These are typically 300–3000 bp long, located upstream of gene coding regions, and usually protected from DNA methylation.

Differentially DNA-methylated region (DMR) A region of DNA that is methylated differentially in the two chromosomes of a diploid cell. These are often associated with genomic imprinting.

DNA methylation A heritable, reversible epigenetic modification in which a methyl group is covalently added to a DNA sequence, usually the fifth carbon of the cytosine pyrimidine ring in a CpG dinucleotide (i.e., 5mC). Recent research is also revealing the presence of non-CG methylation in mammalian cells.

DNA methyltransferase (DNMT) A family of enzymes (DNMT1, 3a, and 3b) that catalyze the addition of a methyl group to cytosine bases.

DNaseI-seq A method that distinguishes open chromatin regions based on their hypersensitivity to DNaseI digestion. Sequencing these genomic fragments can generate genome-wide maps of chromatin accessibility.

Epigenetic mark A modifying moiety that carries an epigenetic signal (e.g., DNA methylation, methylation, and acetylation of histones).

Epigenome All of the epigenetic marks present throughout the genome of a cell.

Formaldehyde-assisted isolation of regulatory elements (FAIRE) Exploits the solubility of open chromatin in the aqueous phase during phenol:chloroform extraction to generate genome-wide maps of soluble chromatin.

High-resolution melting (HRM) analysis A real-time, PCR-based method that relies on the rate of DNA melting reflecting differences in the C:T content determined by the level of methylation.

High-performance liquid chromatography (HPLC) A chromatographic technology used to separate and quantitate mixtures of substances in solution

Histone Chromosomal architectural proteins that bind DNA within nucleosomes. In eukaryotes there are four core histones (H2A, H2B, H3, and H4), the non-nucleosomal linker histone H1, and variant histones.

Histone code The hypothesis that the locations and types of histone modifications, through chromatin remodeling and/or recruitment of transcription factors, predict the effects of those modifications on gene expression.

HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) Methylation-sensitive restriction digested DNA fragments are labeled with fluorescent dyes and co-hybridized to an oligonucleotide microarray.

Imprinting Epigenetic regulation in which maternally and paternally inherited alleles are differentially expressed owing to *cis*-acting modifications of DNA or histones inherited from parental chromosomes.

MBD-seq Identification of methylated DNA by precipitation with a methylated DNA-binding protein specific for 5mC followed by detection of precipitated DNA.

Methylated CpG island recovery assay (MIRA) Using the MBD2/MBD3L1 complex (a methylated DNA–binding protein complex) to enrich methylated DNA fragments for subsequent analysis.

Methylated DNA immunoprecipitation (methyl-DIP or MeDIP) Identification of methylated DNA by precipitation with an antibody specific for 5mC followed by detection of precipitated DNA.

Methylation-dependent restriction enzyme A restriction enzyme that requires DNA methylation for cleavage.

Methylation-sensitive PCR (MSP) Determination of the methylation status of specific DNA sequences by PCR amplification of a converted-converted template with different primer sets that distinguish methylated DNA and non-methylated (C>T converted) DNA.

Methylation-sensitive restriction enzyme A restriction enzyme whose activity is blocked or impaired by DNA methylation.

Methylation-sensitive single nucleotide primer extension (Ms-SNuPE) Determining methylation status of a targeted base bisulfite conversion by primer extension with labeled dCTP or dTTP to distinguish methylated and non-methylated DNA.

MethylC-seq Identification of methylated DNA by shotgun sequencing of bisulfite-converted DNA.

MNase-seq Distinguishes nucleosome positioning based on the ability of nucleosomes to protect associated DNA from digestion by micrococcal nuclease. Protected fragments are sequenced to produce genome-wide maps of nucleosome localization.

Next-generation sequencing (NGS) Highly parallelized DNA-sequencing technologies that produce up to millions of short reads (25–500 bp) at a relatively low cost and in a short time. By contrast, traditional Sanger sequencing produces only a few hundred reads per run.

Nucleosome The repeating unit of chromatin structure; one nucleosome is comprised of 147 bp of DNA wrapped around a protein octamer including two molecules each of the core histones H2A, histone H2B, histone H3, and histone H4.

Reduced representation bisulfite sequencing (RRBS) Sequencing of untreated and treated-DNA fragments that were adapter ligated and size selected after restriction enzyme digestion.

CHAPTER

Laboratory Epigenetic Models of Schizophrenia

8

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KEY CONCEPT

- Laboratory epigenetic models of schizophrenia could help investigate the epigenetics of schizophrenia.

ABBREVIATIONS

5-HIAA 5-Hydroxyindoleacetic acid
BDNF Brain-derived neurotrophic factor
ChIP Chromatin immunoprecipitation assay
CpG Cytosine–phosphate–guanine
DMEM Dulbecco's Modified Eagle Medium
DNMT DNA methyltransferase
DNMTi DNA methyltransferase inhibitor
DOHaD Developmental origins of health and disease
DOPAC 3,4-Dihydroxyphenylacetic acid
E7 Embryonic day 7
FBS Fetal bovine serum
FGF2 Fibroblast growth factor 2
FUDR 5-Fluoro-2′-deoxyuridine
GABA Gamma aminobutyric acid
GAD67 Glutamic acid decarboxylase 67
GFP Green fluorescent protein
GLP G9a-like protein
HDACi Histone deacetylase inhibitor
HMT Histone methyltransferase
HPA Hypothalamic–pituitary–adrenal
HPLC High-performance liquid chromatography
HVA Homovanillic acid
iPSC Induced pluripotent stem cell
LSD *d*-Lysergic acid diethylamide
MeCP2 Methyl-CpG-binding protein 2
MEM Minimal essential medium
mGlu Metabotropic glutamate receptor
MIH L-Methionine-induced hypermethylation
miRNA microRNA
NPCs Neural progenitor cells
NT2 cells Neuronal precursor 2 cells
PRS Prenatal restraint stress
RT-PCR Reverse transcription–polymerase chain reaction
SAH *S*-adenosylhomocysteine
SAMe *S*-adenosylmethionine
SZ Schizophrenia
THC Tetrahydrocannabinol

Introduction

There is increasing evidence that mechanisms regulating gene expression are abnormal in patients with schizophrenia and that dysregulation of epigenetic mechanisms contributes to the pathogenesis of this disorder [1–3]. It is not always possible to conduct investigational or interventional studies in patients with psychiatric disorders such as schizophrenia (SZ) for a variety of reasons: (1) patients with SZ may be unavailable, (2) patients with SZ may be available but unwilling to participate in studies, and (3) the primary site of the human body involving epigenetic dysregulation in SZ, the brain, is highly inaccessible. In the light of these reasons, epigenetic laboratory models of SZ must serve as relevant substitutes for patients with SZ for research purposes. This chapter discusses epigenetic laboratory models of SZ that can be used as possible substitutes for the actual psychiatric disease process in patients (see Box 8.1).

Laboratory non-epigenetic models of schizophrenia

Even before the advent of research on epigenetics in SZ, laboratory models of SZ simulated, as far as possible, the pathophysiology and clinical presentation of the disease. These models are of two types: *in vitro*

BOX 8.1 LABORATORY EPIGENETIC MODELS OF SCHIZOPHRENIA

- L-Methionine-induced hypermethylation (MIH) model
- Prenatal restraint stress (PRS) model: mice and rats
- Prenatal infection models
- Cell-based models
  - Neuronal precursor cells (NT2 cells)
  - Primary cortical and hippocampal neuronal cultures
  - Induced human pluripotent stem cells

models and *in vivo* models. *In vitro* models are typically necessary for hypothesis testing at the cellular level, and *in vivo* models offer an opportunity to test hypotheses in a living organism.

In vitro non-epigenetic models of schizophrenia

In vitro models make use of studies done outside a living organism or natural setting such as a laboratory vessel (e.g., Petri dish, test tube) or other controlled experimental environment. Examples of measurements useful for understanding certain aspects of SZ are the determination of dopamine autoreceptor activity in homogenized tissue obtained from the rat brain [4,5]; determination of dopamine-sensitive adenylyl cyclase activity in the rat striatum [6,7]; simultaneous determination of norepineprine, dopamine, 3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-hydroxyindoleacetic acid (5-HIAA), and serotonin from the rat brain [8,9]; and measurement of levels of neurotransmitters such as dopamine in rats by intracranial microdialysis [10,11]. Results from measurements such as these often lead to testable ideas that are more directly relevant to either potential drug therapies or a better understanding of the pathophysiology of SZ. In addition, *in vitro* models include the use of various cells in culture to examine the biochemistry and physiology of various receptors, as well as the consequences of drugs used in the treatment of psychiatric diseases.

In vivo rodent non-epigenetic models of schizophrenia

With the increased ease associated with experimentally inducing gene knockouts and conditional knockouts in animals, a growing number of SZ risk genes have been manipulated to better understand behaviors associated with known "high-risk" genes. Because these models generally involve the use of mice whose genome has been manipulated in some way, these mice are typical of genetic models of SZ. Mice with mutations can be augmented by studies done to measure various behaviors or the action of drugs on these behaviors such as the production of catalepsy, which is the failure to correct an externally imposed, unusual posture over a prolonged period of time [12,13]; inhibition of apomorphine-induced climbing behavior in mice [14]; and apomorphine-induced stereotypy, which is the frequent, almost mechanical, repetition of the same movement in rats [15].

Mimicking schizophrenia: chronic *d*-lysergic acid diethylamide administration to simulate psychosis

The serendipitous discovery of the hallucinogenic properties of *d*-lysergic acid diethylamide (LSD) in 1943 by Albert Hoffman in the laboratories of Sandoz (now Novartis) spawned several decades

of research by biological psychiatrists attempting to better understand psychosis. Moderate doses of LSD (75–150 μg) produce perceptual changes, including illusions, hallucinations, synesthesias, and altered perception regarding the passing of time. These alterations in perception are typically intense and generally interfere with cognitive testing. The clinical effects of LSD are biphasic; an initial hallucinatory experience in the early phase gives way to a paranoid phase reminiscent of amphetamine-induced psychosis in humans [16]. LSD has been used for some 50 years for drug discrimination studies to better understand its pharmacological mechanism of action [17]. Although pharmacological research has produced some 10,000 papers on LSD, its mechanism of action is still not completely understood [18]. These studies have included an examination of the tissue distribution, toxicology, plasma levels, metabolism, excretion, and neurophysiological actions [18].

Population-wide studies of hospital records indicate that substance-induced psychotic disorders predict SZ spectrum disorders to a greater extent than originally thought [19]. LSD is believed to mimic some aspects of the positive symptoms (visual hallucinations) of psychosis, but other compounds (including stimulants and cannabinoids such as tetrahydrocannabinol, or THC) elicit various phenotypes including paranoid thoughts [20]. Chronic administration of low doses of LSD to rats induces hyperactivity, hyperirritability, increased locomotion, anhedonia, and impaired social interaction [17]. These symptoms resemble those seen in humans with psychosis. Chronic LSD administration also induces changes in gene expression related to multiple neurotransmitter-related genes, including those related to dopamine and serotonin. Interestingly, the symptoms could be transiently relieved by haloperidol and olanzapine. Mice treated chronically with clozapine showed attenuated behavioral responses to single doses of LSD [21], and this is thought to occur due to the downregulation of 5HT2A receptors. The use of psychotropic drugs and psychostimulants has tremendous research potential as a means of modeling certain aspects of SZ in animals.

In vivo epigenetic models of schizophrenia

L-methionine-induced hypermethylation (MIH) model of schizophrenia

Based on numerous reports published between 1961 and 1971 on the recrudescence of symptoms induced by a two-week treatment of high daily doses of L-methionine (20–40 g) to patients with SZ, Tremolizzo et al. [22] reported a L-methionine-induced hypermethylation (MIH) model of schizophrenia. The authors administered L-methionine (1 g/kg, 6.6 mM/kg) subcutaneously, twice daily, to 60-day-old mice (B6C3Fe strain, The Jackson Laboratory) for 15 consecutive days. Control mice received only the vehicle. Twelve hours after the last injection, animals were sacrificed and their brains were immediately frozen at −80°C. Total RNA was later extracted from the frontal cortex of the mouse brain. Reelin and glutamic acid decarboxylase 67 (GAD67) mRNA content was measured using a quantitative reverse transcription–polymerase chain reaction (RT-PCR) assay. Frontal cortical samples were also homogenized and cellular protein was extracted. Reelin and other proteins were detected immunologically using western blot analysis. DNA (2 μg) was also isolated from frontal cortex tissues and analyzed for promoter methylation status of the *RELN* gene. Levels of *S*-adenosylmethionine (SAMe) and its demethylated metabolite *S*-adenosylhomocysteine (SAH) in homogenized frontal cortex samples were determined by high-performance liquid chromatography (HPLC). More details about the methodology of the MIH model are provided in the original paper regarding the study designed to replicate human findings in an animal model [22]. The study

reports that L-methionine-treated mice showed significant downregulation of the expression of both *RELN* and GAD67 mRNAs (by about 40%) compared with control mice. There also was a significant decrease in the levels of both 400- and 180-kDa reelin immunoreactive proteolytic fragments in the L-methionine-treated mice. The levels of SAMe in the frontal cortex increased by 50% in the treated mice. This rise was found to persist for at least 4 hours. SAH levels were also found to be raised in the mice treated with L-methionine. In a cytosine–phosphate–guanine (CpG) dinucleotide island that overlapped the promoter of *RELN* there was a significant increase in the number of methylated cytosines. The mean cytosine methylation levels significantly correlated with the levels of *RELN* mRNA. It was also noted that L-methionine treatment disrupted prepulse inhibition of startle reflex, a laboratory animal behavior associated with SZ. This suggested abnormalities in sensorimotor gating. Moreover, it was found in this study that sodium valproate, at doses that increase acetylated histone content, normalized L-methionine-induced downregulation of reelin and GAD67 mRNA.

In a follow-up to the original report, Tremolizzo et al. [23] investigated in some detail the behavioral effects of protracted L-methionine administration to mice. The behaviors reported by the authors were social interaction in the home cage, social interaction in a novel environment, social isolation-induced aggression, prepulse inhibition of startle reflex, and locomotor activity. The authors found that, compared with control mice, L-methionine-treated mice show reduced social interaction in the home cage, reduced social interaction in a novel environment, decreased aggression due to social isolation, and impaired prepulse inhibition of the startle reflex. Locomotor activity was not significantly affected in the mice that received L-methionine treatment when compared with control mice.

Assessment of L-methionine-induced hypermethylation model of schizophrenia

The findings reported by Tremolizzo and colleagues [22] showed that L-methionine induces a downregulation of the expression of RELN and GAD67 mRNAs in the frontal cortex of mice. This result has relevance to SZ because a similar phenomenon has been observed in the postmortem brain of patients with SZ [1,3]. The MIH model also shows similarities to SZ in that when patients with SZ are administered L-methionine at high doses daily for a protracted period, they experience an aggravation of symptoms. There are indeed behavioral similarities between the MIH model and SZ; thus, L-methionine-treated mice exhibited a suppression of social interaction [23], a behavioral pattern that simulates social withdrawal in humans with SZ. The impaired prepulse inhibition of startle reflex shown by L-methionine-treated mice simulates impaired sensory gating in SZ patients, which leads to attention and cognition deficits.

The MIH model of SZ also shares biochemical similarities with SZ; thus, postmortem brain of patients with SZ is characterized by a decrease in the density of spines on layer III pyramidal neuron dendrites in the prefrontal cortex [24]. This is an area of the brain in which RELN and GAD67 levels are decreased in distinct populations of GABAergic neurons [25,26]. In this context, Tueting et al. [27] sought to determine whether L-methionine-treated mice show a decrease in the dendritic spine density of layer III pyramidal neurons in the frontal cortex. These authors found that after both 7 and 14 days of L-methionine treatment there was a significant reduction of spine density in pyramidal neurons in layer III of the frontal cortex of the mice treated with L-methionine, compared with control mice that received only vehicle. Moreover, after 7 days of administration, withdrawal of L-methionine for 12 days caused a recovery of spine density on the dendrites to the levels of vehicle-treated mice. It was also found by the authors that concurrent administration of L-methionine and sodium valproate

(2 mM/kg subcutaneously twice daily for 7 days) prevented the decrease in density of spines. Collectively, these findings illustrate the utility of the MIH model in mimicking various aspects of the SZ phenotype and in the testing of new drugs that might prevent the onset of these symptoms.

Prenatal restraint stress model for schizophrenia

There is growing evidence that the prenatal environment influences the newborn phenotype. The effects of the prenatal environment, and particularly stress, can have lifelong effects on the offspring. Indeed, this is implicated in the developmental origins of health and disease (DOHaD) hypothesis [28,29]. During critical periods of prenatal and postnatal mammalian development, environmental factors, especially stress, influence developmental pathways and can hence induce permanent changes in cognition and susceptibility to psychiatric disease. There is evidence that epigenetic mechanisms of gene expression underlie this phenomenon [29,30]. The DOHaD hypothesis has been supported by a worldwide series of epidemiological studies. In this context, prenatal stress has been shown to have abnormal cognitive, behavioral, and psychosocial effects in both animals and humans [31–34].

Prenatal stress can be induced in several ways in rodents: saline injection, forced swimming, crowding, and, most often, restraint alone or restraint with heat and bright light [33]. In non-human primates, an acoustic startle protocol is preferred [33]. The common feature among these stressors is that they are relatively unpredictable and uncontrollable events, with sudden onsets that trigger a hypothalamic–pituitary–adrenal (HPA) response in the animals [33]. However, the level of stress response in the pregnant animal is thought to depend on the type of stressor. In the pregnant dam, it has been shown that restraint, forced swimming, and crowding each significantly increase plasma levels of corticosterone [35]. However, the increase in stress varies by stressor, with restraint showing the greatest increase, followed by forced swimming, and finally by crowding. The effects of prenatal stress are also known to depend on other factors, such as sex of the offspring [32,36], time of gestation when the stressor is applied [33], and species and strain of animals [33,37]. Regarding the mechanism of prenatal stress on the offspring, epigenetic mechanisms are known to be involved [32], as well as various changes in neurotransmission in the brain such as those involving GABA [38], glutamate [39], and corticosteroid hormones [31].

Prenatal restraint stress model in mice

Matrisciano et al. [40] developed and characterized an experimental mouse model in offspring of mothers restrained during gestation as a possible laboratory model of SZ. These workers housed pregnant mice individually with a 12-hour light/dark cycle with food and water *ad libitum*. Control dams were left undisturbed throughout gestation, whereas stressed dams were subjected to repeated episodes of restraint stress. The stress procedure restrained the pregnant dam in a 12 × 3-cm transparent tube under a bright light for 30 minutes two times a day from the seventh day of pregnancy until delivery. Following weaning (postnatal day 21), male mice were chosen for further study and housed separately, four per cage. After sacrificing the mice, total RNA was extracted from the mouse frontal cortex. RT-PCR was performed to measure the mRNA levels of metabotropic glutamate receptor 2 (mGlu2), metabotropic glutamate receptor 3 (mGlu3), brain-derived neurotrophic factor IX (BDNF-IX), and GAD67. Western blot analysis for mGlu2 and mGlu3 receptors, DNA methyltransferase 1 (DNMT1),

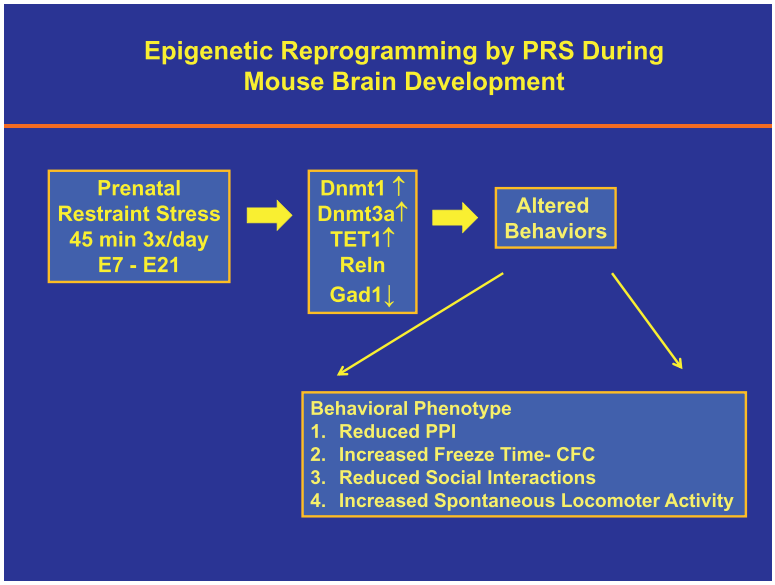

FIGURE 8.1

Schematic diagram showing some effects of prenatal restraint stress in mice.

GAD67, and methyl-CpG-binding protein 2 (MeCP2) was performed. DNMT1 binding to the mGlu2 and mGlu3 promoters was measured using chromatin immunoprecipitation (ChIP). MeCP2 binding to mGlu2, mGlu3, GAD67, and BDNF-IX promoters was measured. Behavioral tests were performed to investigate social interaction, locomotor activity, and prepulse inhibition of startle reflex. The authors found that mice subjected to prenatal restraint stress (PRS) showed reduced expression of both mGlu2 and mGlu3 receptors in the frontal cortex from the first day of postnatal life. Such mice also showed changes in gene expression of the BDNF, GAD67, and DNMT1 mRNAs. The authors also demonstrated abnormalities in social interaction, locomotor activity, and prepulse inhibition of the startle reflex in these mice. Some of these features are shown in Figure 8.1. The authors suggested that these features represent behavioral and biochemical changes consistent with a SZ-like phenotype.

Prenatal restraint stress model in rats

Maccari et al. [41] described PRS in rats. The authors randomly assigned pregnant Wistar rats weighing 250 g to PRS and control groups, individually housing them with *ad libitum* access to food and water. A constant light/dark cycle (on at 6:00 hours; off at 20:00 hours) was maintained and temperature (22°C) and humidity (60%) were kept constant. The restraint stress was performed similarly between the 14th and 21st days of pregnancy. These sessions were performed in a lighted environment. This stress procedure was chosen because it was known to have an indirect influence on the fetus via a direct stressful influence on the mother. At birth, half of the pups were raised by their biological mothers and the other half were assigned to either control or prenatally stressed

foster mothers. Pups were briefly placed in the cage with the adoptive mothers within the first 3 to 6 hours of birth. During this procedure, the mothers were briefly (less than 1 minute) removed from their cages. The pups were weaned 21 days after birth and left undisturbed until the time of testing, at 90 days of age. The authors found that in adults PRS prolonged corticosterone secretion induced by restraint stress. Corticosterone levels in either basal conditions or 30 minutes after stress did not differ between the control and PRS rats. PRS also decreased hippocampal corticosteroid receptors. Adoption at birth totally reversed the effects of PRS on both corticosterone secretion and hippocampal receptors. Adoption also increased maternal behavior in that foster mothers spent longer time licking and picking up pups than did biological mothers. The latency to replace all the pups in the nest was also lower with the foster mothers than with the biological mothers. Thus, this study indicated that prenatal and postnatal events have a long-term effect on the HPA axis.

Assessment of prenatal restraint stress as an epigenetic model of schizophrenia

Several studies have made use of the PRS model of SZ in mice and rats to investigate the role of epigenetics in the pathophysiology and treatment of SZ. For example, Koenig et al. [42] used the PRS model in Sprague–Dawley rats and found elevated plasma glucocorticoid levels in the adult offspring, suggesting reduced glucocorticoid negative feedback. The authors also found an enhanced locomotor response to amphetamine on postnatal day 56 and a decrease in prepulse inhibition of the startle reflex across a range of prepulse stimulus intensities. In addition, PRS-exposed rats showed evidence of a disruption in auditory sensory gating as measured by the N40 response. The authors suggest that the behavioral effects of PRS in the offspring may be useful for learning aspects of the pathophysiology of SZ, as the behaviors that were altered have been used to validate animal models of SZ.

Later, Morgan and Bale [43] used the PRS model in mice to determine epigenetic programming effects and the transmission of a dysmasculinized phenotype to second-generation male offspring via the paternal lineage, focusing on an analysis of the transcriptional regulation of the miRNA environment with broad impact on developmental processes. The authors examined programming effects in second-generation offspring of prenatally stressed (F2-S) or control (F2-C) sires. Examination of gene expression patterns during the perinatal sensitive period, when organizational gonadal hormones establish the sexually dimorphic brain, confirmed dysmasculization in F2-S males, where genes important in neurodevelopment showed a female-like pattern. Analyses of the epigenomic miRNA environment found reductions in miR-322, miR-574, and miR-873 in the F2-S male brain, levels that were more similar to those of control females. Increased expression of a common gene target for these three miRNAs was confirmed in the males. These developmental effects were associated with the transmission of a stress-sensitive phenotype and shortened anogenital distance in adult F2-S males. As confirmation that the miRNA environment responds to organizational testosterone, neonatal males that received formestane, an inhibitor of aromatase, the enzyme that catalyzes the conversion of androgens to estrogens, showed dramatic changes in brain miRNA patterns. This suggested that miRNAs can serve a role in organizing the sexually dimorphic brain. The authors suggested that their data support the existence of a sensitive period of early gestation when epigenetic programming of the male germ line can occur, allowing the transmission of specific phenotypes to later generations. The authors also suggested that their data can provide insight into sensitive periods and sex-specific vulnerability to the etiology of neurodevelopmental disorders such as SZ.

More recently, Matrisciano et al. [38], applying the PRS model in mice, found that offspring born from non-stressed mothers (control mice) had high levels of DNMT1 and 3a mRNA expression in

the frontal cortex at birth, but these levels progressively declined at postnatal days 7, 14, and 60. Offspring born from stressed mothers (PRS mice) showed raised levels of DNMTs in comparison to controls at the time points studied, including birth (P0), and on postnatal day 60. Using GAD67–green fluorescent protein (GFP) transgenic mice, the authors established that in both control and PRS mice increased levels of DNMT1 and 3a were preferentially expressed in GABAergic neurons of the frontal cortex and hippocampus. The overexpression of DNMTs in GABAergic neurons was associated with a decline in reelin and GAD67 expression in PRS mice during early and adult life. PRS mice also showed increased promoter binding of DNMT1 and MeCP2, and an increase in 5-methylcytosine and 5-hydroxymethylcytosine in specific CpG regions of the promoters of the *RELN* and *GAD1* genes. Behaviorally, adult PRS mice showed hyperactivity and deficits in social interaction, prepulse inhibition of startle reflex, and fear conditioning that were corrected by the administration of valproic acid or clozapine. In the light of these findings, the authors suggested that PRS in mice induces abnormalities in the DNA methylation network and in behavior suggestive of an SZ-like phenotype.

Mice and rats subjected to PRS develop characteristics resembling SZ, as described above. However, PRS also produces characteristics in mice and rats that resemble other psychiatric disorders such as attention deficit hyperactivity disorder, major depressive disorder, anxiety disorder, cognitive disorders, and drug addiction [33,44]. In this light, the PRS model in mice and rats may not be specific for SZ.

Prenatal infection models

There is a considerable body of evidence to support the concept that environmental factors including pre- and perinatal stress, difficulties during birth (prolonged labor, oxygen deprivation), nutritional deficiencies, and viral infection (immune activation) all contribute to the genesis of SZ and autism-like disorders [30]. While these factors may differ in terms of the modes of action, perturbations in circulating glucocorticoids due to stress or the production of cytokines in response to inflammation underlie many of the subsequent outcomes. The prenatal immune challenge to pregnant rodents produces abnormalities in the behavioral phenotype, histological data, and mRNA expression [45]. Maternal viral infection causes SZ-like alterations of 5-HT_2A and $mGlu_2$ receptors in the adult offspring [46]. The offspring of mice prenatally infected with influenza at times from E7 through E18 show alterations in gene expression and protein and brain structural abnormalities [47]. Moreover, the synthetic viral mimic Poly(I:C) and the proinflammatory agent lipopolysaccharide also demonstrate brain and behavioral abnormalities in offspring. Because SZ is a multifaceted disorder with complex clinical presentation, no single prenatal stressor is likely to provide an opportunity to study the many phenotypes that dominate the disease. It seems likely that by examining the phenotype generated by each of the above models, a composite picture may form that will allow the assignment of particular sets of genes to particular behaviors and abnormalities.

In vitro epigenetic cell-based models of schizophrenia

Epigenetic models of schizophrenia based on cell culture

Understanding neurons at the cellular and molecular levels is the cornerstone of neuroscience. To this end, neuronal culture plays an important role. The complexities of neuron structure and function require unusual methods of culture in order to determine how abnormalities in or between cells in the

brain give rise to brain dysfunction and disease. Studies of neurons in culture have been going on for over a century, beginning in 1910, a field well reviewed by Millet and Gillette [48]. Various neuronal culture-based models have been used to investigate the role of epigenetics in the pathophysiology and treatment of SZ. Although other models are available, here three models based on neuronal culture are described: neuronal precursor cells, primary cortical neuronal culture, and primary hippocampal neuronal culture.

NT2 neuronal precursor cells

NT2 neuronal precursor cells are a cell line derived from a human male germ cell carcinoma. This cell line is one of the best documented stem cell lines that can be made to differentiate into neurons and astrocytes. The cell line will differentiate into neuronal cells by incubation with retinoic acid [49]. Chen et al. [50] maintained NT2 cells in Dulbecco's Modified Eagle Medium/F-12 nutrient mixture (DMEM/F12), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and glutamine. Differentiated (hNT) neurons were induced from cultures of NT2 cells by treating with retinoic acid for up to 6 weeks. Low-density cultures of NT2 neural progenitors were treated with the DNA methyltransferase inhibitor (DNMTi) decitabine at several concentrations (1, 5, and 10 μ*M*) and the histone deacetylase inhibitor (HDACi) trichostatin A (0.2, 2,5, and 10 μ*M*) for various times; cells were harvested for total RNA isolation. In addition, NT2 cells were treated with various concentrations of valproic acid (0.2, 2, 5, and 10 m*M*) and the inactive valproic acid amide valpromide (5 m*M*) for 40 hours and then harvested for analysis of RNA. The authors showed that the *RELN* promoter is more heavily methylated when the gene is silent. Activation of the *RELN* gene by various agents such as retinoic acid, decitabine, valproic acid, and trichostatin A correlated with a decrease in *RELN* promoter methylation. Later, Kundakovic et al. [51] investigated the effects of the DNMTi doxorubicin, azacytidine, and zebularine on reelin and GAD67 expression in NT2 cells. Competitive RT-PCR with internal standards was used to quantitate mRNA levels. The authors found that reelin and GAD67 mRNAs were induced in a dose-and time-dependent manner by these drugs. The authors also provided evidence that the transcription of the *RELN* gene is repressed by the DNA methylation-mediated recruitment of DNMT1, MeCP2, and possibly other co-repressors, including HDACs. The two studies discussed above [50,51] have relevance to the epigenetics of SZ because epigenetic changes in the *RELN* gene have been implicated in the pathogenesis of SZ [1,3].

Primary neuronal cultures

Primary neuronal cultures lack a genetically defined, layered, or cell nucleus topology. Despite this, however, such cultures share many features with the tissues from which they are obtained [52]. Discussed below are two types of primary neuronal cultures: primary cortical neuronal culture and primary hippocampal neuronal culture.

Primary cortical neuronal culture

As mentioned above, the chronic administration of L-methionine to patients previously diagnosed with SZ precipitated a recrudescence of symptoms. To investigate this observation *in vivo*, mice were treated chronically with L-methonine as described (see MIH model of SZ). To study the biochemical

mechanism *in vitro*, Noh et al. [53] prepared primary cultures of neurons obtained from neocortices removed from fetal mice (embryonic days 14 to 15). The neocortices were dissociated and plated in minimum essential medium (MEM) supplemented with 5% horse serum, 5% FBS, and 21-m*M* glucose. The proliferation of non-neuronal cells was prevented using 10-μ*M* cytosine arabinoside at 7 to 8 days *in vitro*, and cultures were incubated with 2-m*M* L-methionine in MEM at 37°C for 24, 36, and 72 hours. Cultures were harvested for both the isolation of genomic DNA and total RNA. The authors showed that similar to observations made *in vivo* in mouse, L-methionine treatment decreased *RELN* mRNA levels and increased corresponding promoter methylation levels [53]. To tie the observed hypermethylation to DNMT1 expression, a DNMT1 antisense RNA was added to the cultures during the L-methionine incubation. The authors showed through quantitative western blot analysis that the antisense RNA downregulated DNMT1 protein levels. Moreover, the authors also demonstrated that the DNMT1-antisense treatment prevented the L-methionine-mediated downregulation of *RELN* mRNA levels. The latter observation was accompanied by decreased *RELN* promoter methylation, thus linking DNMT1 to the L-methionine treatment. Although these experiments are not definitive, they strongly imply that promoter hypermethylation might be associated with the L-methionine-induced reappearance of SZ symptoms previously reported [54].

More recently, Chase and Sharma [55] administered nicotine to mice intraperitoneally. Histone methyltransferase (HMT) mRNA and H3K9me2 levels were estimated in primary cortical neuronal cultures and cortices extracted from the mice, as well as in human lymphocyte culture. Bdnf/BDNF mRNA levels were examined as an epigenetically regulated read-out of gene expression. There was a significant decrease of the HMT G9a-like protein (GLP), G9a, and Setdb1 mRNA expression in the nicotine-treated tissues studied, with significant decreases observed in both total and promoter-specific H3K9me2 levels. Increasing doses of nicotine caused significant decreases in Bdnf/BDNF promoter-specific H3K9me2 binding, resulting in increased Bdnf/BDNF transcription. The authors inferred that their data suggest that nicotine reduces markers of a restrictive epigenomic state, hence leading to a more permissive epigenomic environment. This study is relevant to the epigenetics of SZ because there is a relatively very high use of nicotine among patients with SZ [56].

Primary hippocampal neuronal culture

Brewer [57] developed and described a technique for the isolation and culture of rat hippocampal neurons. For each experiment, the author used a single male Sprague–Dawley, Fischer 344, or F1 hybrid of Fischer 344×Brown Norway rat which was anaesthetized with ether and then sacrificed. The hippocampi were rapidly dissected out from the brain in 2 mL Hibernate®/B27 at 4°C in a 35-mm-diameter Petri dish. Hibernate contained common salts, glucose, pyruvate, 17 amino acids, and 9 vitamins and was buffered for use with adult neurons at ambient CO_2. Hibernate was further supplemented with 0.5-m*M* glutamine. B27 was a serum-free medium supplement containing 20 ingredients. Meninges and excess white matter were removed. Hippocampi were then transferred onto sterile filter paper prewet. Slices of 0.5 mm thickness were cut perpendicularly to the long axis of the hippocampi and transferred to a tube containing Hibernate/B27. After being shaken for 8 minutes at 30°C the slices were transferred to another tube at 30°C containing papain. Slices were incubated for 30 minutes in a 30°C bath and transferred to a 15-mL tube containing 2 mL Hibernate/B27 at 30°C. After 5 minutes at room temperature, the slices were triturated and allowed to settle, and the supernatant was transferred to another tube. Twice more, the sediment was suspended in

2 mL Hibernate/B27, triturated 10 times, and allowed to settle, and the supernatants were combined. The result was a cell suspension in 6 mL Hibernate/B27. Using a gradient of NycoPrep™ 1.15 (Life Technologies #100-2420) or 1 mL 4% bovine serum albumin in Hibernate/B27 in a 15-mL centrifuge tube, cells were isolated. The cells were then plated on glass cover slips previously coated with 50 µg/mL poly-D-lysine in water. The polylysine was applied overnight, aspirated, rinsed with water, and allowed to dry. One hour after plating and incubation, unattached cells and debris were drained as the cover slip was quickly transferred to 0.4 mL B27/Neurobasal® in a 24-well plate at 37°C. The medium was aspirated to remove more debris, rinsed with warm Hibernate, and replaced with 0.4 mL growth medium, B27/Neurobasal, with 0.5-m*M* glutamine, no glutamate, 50 units penicillin, 0.05 µg/mL streptomycin, and 5 ng/mL fibroblast growth factor 2 (FGF2). More details of the culture method are given in the original paper [57].

More recently, Ehrenreich et al. [58] cultured rat hippocampal neurons in order to investigate the application of erythropoietin as a neuroprotective agent in SZ. The authors showed that the haloperidol-induced rise in death rate of cultured rat hippocampal neurons was significantly reduced by the addition of recombinant human erythropoietin. These results contributed to the authors' suggestion that recombinant human erythropoietin could be a useful compound for neuroprotective add-on strategies in SZ.

In addition to rat hippocampal neurons, mouse hippocampal neurons can also be cultured in *in vitro* studies into the role of epigenetics in schizophrenia. Mayer et al. [59] have described the culture of these cells. These authors prepared primary dissociated glial cell cultures by dissociating the hippocampi of newborn C57B1/6 mice and plating the resulting cell suspension at 2.5×10^5 cells per 35-mm Petri dish. Before use, the Petri dishes were coated with collagen and poly-L-lysine. Glial cell cultures were grown in DMEM with added bicarbonate, 10% FBS, and 1% penicillin–streptomycin using a 90% air and 10% CO_2 atmosphere maintained at 35.5°C.

When cultures became confluent, the medium was changed to MEM with 5% horse serum, and 5-fluoro-2′-deoxyuridine (FUDR) and uridine were added for 5 to 7 days to arrest cell division. To prepare neuronal cultures, the authors dissected the hippocampus from 16- to 17-day C57B1/6 mouse embryos, dissociated them, and plated them at 12,500, 25,000, or 10^5 cells per dish, as required, onto the glial cell feeder layer cultures. Before removal of the fetuses, the mothers were killed by cervical dislocation. The medium was comprised of MEM, 5% horse serum, and a nutrient supplement containing transferrin, insulin, selenium, corticosterone, triiodothyronine, progesterone, and putrescine. No antibiotics were added. One day after plating, the cultures were given a complete change of medium, and FUDR and uridine were added for 5 to 7 days to suppress glial cell division. More details of the culture method are given in [59]. Bharadwaj et al. [60] used primary hippocampal neuronal culture to demonstrate upregulation of glutamic acid decarboxylase 1 (GAD1) as part of multiple lines of evidence that dynamic regulation of three-dimensional genome structure plays a critical role for orderly development and functioning of GABAergic systems in the human brain. The study by Bharadwaj et al. [60] has relevance to the epigenetics of SZ because epigenetic changes in the GAD1 gene have been implicated in the pathogenesis of SZ [1,3].

Induced human pluripotent stem cells

Differentiated human cells can be induced to become pluripotent stem (iPS) cells. This pioneering, recently developed technology [61] provides a powerful tool for disease modeling. The advantages

of iPS cells in modeling disease are that (1) they are directly derived from adult patients; (2) this technology allows for the study of specific cell types of diseases as iPS cells, being self-renewing and pluripotent, can be induced into any cell type in the body; and (3) patient-derived iPS cells maintain cellular defects not observed in iPS cells from healthy controls. Recently, Brennand et al. [62] modeled the cellular dysfunction in schizophrenia using fibroblasts from SZ patients. Briefly, fibroblasts from control and SZ subjects were reprogrammed with tetracycline-inducible lentiviruses that express select transcription factors, including OCT4, SOX2, KLF4, cMYC, and LIN28 [62]. The cells were reprogrammed for 21 to 28 days in HUES medium. iPS cell colonies were picked and lines were maintained on mouse embryonic fibroblasts in HUES medium or on Matrigel™ in TeSR™ medium. For differentiation of iPS cells to neural progenitor cells (NPCs) and neurons, embryoid bodies generated from iPS cells were transferred to non-adherent plates. Colonies were maintained in N2 medium and plated onto polyornithine/laminin-coated plates. The visible rosettes formed were manually dissected and cultured in NPC medium. Neural differentiation was made by dissociating NPCs with Accutase® and plating at low density in neural differentiation medium. With these induced iPS cell neurons, the assays for neuronal connectivity, neurite outgrowth, synaptic density, electrophysiology, and gene expression were conducted [62].

Assessment of epigenetic cell-based models of schizophrenia

The cell-based epigenetic models, unlike the MIH model and the PRS animal models, are *in vitro* models. The cell-based models could prove to be useful in the study of the role of epigenetics in the pathophysiology and treatment of SZ. For example, these models could be useful in studying the effects of existing drugs for treating SZ, as well as new drugs for treating SZ, on epigenetic mechanisms of gene expression in neurons. In addition to SZ, cell-based models could also be useful in investigating the role of epigenetics in the pathophysiology and treatment of other psychiatric disorders such as bipolar disorder, major depressive disorder, and cognitive disorders such as Alzheimer's disease.

Conclusions

It is well known that among laboratory models of diseases, laboratory models of psychiatric diseases such as SZ are the most difficult to develop and validate [63]. The laboratory models of SZ described in this chapter could help in the investigation of the role of epigenetics in the pathophysiology and treatment of SZ. However, work on laboratory epigenetic models of SZ is in its early stages. As discussed by Nestler and Hyman [63], laboratory non-epigenetic models of SZ have not delivered in terms of predicting therapeutic efficacy in humans of effective drugs with novel mechanisms of action. The laboratory epigenetic models of SZ described in this chapter, as well as forthcoming such models, may do better in this regard and help usher in the development of newer and better drugs for the treatment of SZ. Chance and serendipity have played a role in the discovery of the first effective drugs for SZ such as chlorpromazine and haloperidol [64,65]. Laboratory epigenetic models of SZ currently available and those that will be developed in the future may help in the rational design and development of new drugs for the management of SZ.

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Glossary

ad libitum Unlimited access.
Apomorphine A non-selective dopamine agonist.
Dam Female parent.
GAD67–GFP transgenic mice Transgenic mice that can be useful for fluorescent labeling of neurons and the study of neurons in the hippocampus.
GLP, G9a Key histone lysine methyltransferases for H3K9me1 and H3K9me2.
In vitro Outside the body.
In vivo Inside the body.
L-Methionine An essential amino acid that is the metabolic precursor of *S*-adenosylmethionine.
Setdb1 An H3K9 histone methyltransferase.
Sire Male parent.

CHAPTER

9

Animal Models of Environmental Manipulations Resulting in Epigenetic Modifications That Increase Risk for Affective Disorders

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KEY CONCEPTS

- Environmental challenges during the prenatal period, such as maternal depression or stress, and maternal exposure to toxins and nutritional deficiencies can result in epigenetic modifications that increase risk for developing mood disorders in adulthood.
- Quality of maternal care and bonding during the postnatal period can influence risk for mood disorders in adulthood through epigenetic modifications.
- Exposure to stressful events, especially social stress, as well as naturally occurring hormonal changes in adulthood can result in epigenetic changes that increase risk for mood disorders.

ABBREVIATIONS

5-HT Serotonin
ABN Arched back nursing
ACTH Adrenocorticotropic hormone
ADHD Attention deficithyperactivity disorder
AVP Arginine vasopressin
BDNF Brain-derived neurotrophic factor
BPA Bisphenol-A
CC Collaborative Cross
Cdkn1c Cyclin-dependent kinase inhibitor 1c gene
Cdkn3 Cyclin-dependent kinase inhibitor 3 gene
CORT Corticosterone
CRH Corticotropin-releasing hormone
CS Conditioned stimulus
CVS Chronic variable stress
D_1 Dopamine receptor subtype 1
D_2 Dopamine receptor subtype 2
DA Dopamine
DNMT DNA methyltransferase
$GABA_A$ Gamma aminobutyric acid A
Gadd45b Growth arrest and DNA-damage-inducible 45 beta gene
GR Glucocorticoid receptor
HPA Hypothalamic–pituitary–adrenal
HR High responder
IUGR Intrauterine growth restriction
LG Licking and grooming
LR Low responder
MeHg Methylmercury
NAc Nucleus accumbens
NGFI-A Nerve growth factor inducible A
NMDAR *N*-methyl-D-aspartate receptor
Nr3c1 nuclear receptor subfamily 3, group C, member 1
NRSF Neuron-restrictive silencer factor
PFC Prefrontal cortex
PVN Paraventricular nucleus of the hypothalamus
RI Recombinant inbred
RIX Recombinant inbred intercrosses
SGA Small for gestation age
SSRI Selective serotonin reuptake inhibitor
T3 Triodoithyronine
TALEs Transcriptional activator-like effectors
US Unconditioned stimulus

Mood disorders

In the United States, the lifetime prevalences of affective disorders such as depression and anxiety disorders are approximately 21% and 29%, respectively [1], and it is estimated that 11% of Americans ages 12 and older are taking an antidepressant [2]. The National Institute of Mental Health-funded Sequenced Treatment Alternatives to Relieve Depression study evaluated the effectiveness of

antidepressants and reported remission rates of 31% after 14 weeks and 65% after 6 months [3]. This study, along with others, shows that there are still a relatively high number of patients are not responsive to antidepressant treatment [3]. Additionally, it can take 3 to 4 weeks after starting antidepressant therapy to feel relief of symptoms [3]. Furthermore, common side effects such as headaches, nausea, drowsiness, agitation, dry mouth, constipation, bladder problems, and sexual dysfunction can often result in cessation of antidepressant use before the drug has had time to exert therapeutic efficacy [4,5]. These problems highlight the need for more effective treatments of mood disorders, and to do this we must first understand the etiology of these disorders.

Several hypotheses exist regarding the etiology of mood disorders. The monoamine hypothesis suggests that decreased function of monoamines such as serotonin (5-HT), dopamine (DA), and norepinephrine can result in the development of depression, anxiety, or schizophrenia. This hypothesis is supported by the antidepressant action of drugs that increase the amount of monoamines in the synaptic cleft, such as selective serotonin reuptake inhibitors (SSRIs). There is also the idea that disruption of limbic brain regions that mediate emotion and reward can also lead to the development of these disorders. Another proposed mechanism is dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis. The HPA axis is a major part of the neuroendocrine system and is responsible for mediating responses to stress. Dysregulation of the HPA axis includes an increase in glucocorticoids, hypersensitivity to adrenocorticotropic hormone (ACTH), increased secretion of corticotropin-releasing hormone (CRH), and impaired glucocorticoid-mediated negative feedback [6–8]. Brain-derived neurotrophic factor (BDNF) has also been implicated in neuropsychiatric diseases. Animal studies have shown that BDNF is reduced in the hippocampus after stress and that antidepressant treatment results in increased BDNF [9]. Collectively, these mechanisms are all likely to contribute to the development of affective disorders, but the extent to which each is involved and the underlying mechanisms responsible are unclear. Furthermore, it is widely acknowledged that there is a significant genetic component for development of affective disorders in combination with environmental risk factors. The interaction of genetic background and the environment can result in epigenetic changes that will persist through the lifespan to increase risk for development of an affective disorder. Common epigenetic changes that can occur in response to environmental challenges are DNA methylation and histone acetylation.

In order to study epigenetic mechanisms, it is necessary to examine specific environmental challenges capable of modifying gene expression and the developmental time points at which the genome is susceptible. Environment, in this sense, is used in the broadest of terms. It refers to toxicological, nutritional, social and other stressors, and hormonal challenges that individuals are exposed to during their lifetime. In this chapter, we will focus on environmental exposures across the lifespan.

This chapter will (1) outline environmental challenges that occur in the perinatal (prenatal and postnatal) period and in adulthood that have been shown to increase risk for mood or anxiety disorders, (2) reports behavioral outcomes in animal models exposed to environmental challenges, and (3) discuss proposed epigenetic mechanisms responsible for behavioral changes.

Rationale for the use of animal models

Human studies to identify biomarkers for anxiety and mood disorders are complicated by a variety of factors, including the lack of experimental control over a subject's environment and genetic background and inaccessibility of brain tissue required for analysis. Furthermore, human studies of

environmental manipulations that result in epigenetic changes are ethically unfeasible during the perinatal period, when many epigenetic modifications are thought to occur. These studies are also hindered by the impracticality of tracking offspring from the time of gestation until the development of psychiatric disorders in early adulthood. Finally, studying the effect of environmental challenges in adults is confounded by unknown previous exposures. The use of animal models, particularly rodents, provides a complementary approach for understanding the processes by which environment and genetic predisposition interact to cause epigenetic changes that can result in the development of affective disorders in adulthood.

There are several advantages to using rodent models to assess the epigenetics of psychiatric illness, including access to brain tissue, ability to control the environment, defined genetic background, shorter gestation period, shorter lifespan, access to advanced genomic tools, and cost-efficiency. The obvious drawbacks, of course, are the substantial differences in developmental periods during gestation and interpretation of rodent behaviors with regard to human symptoms. However, rodents recapitulate many of the endocrine and HPA axis changes observed in humans during gestation and the postpartum period, and many of the same neuropeptides and hormones that promote maternal care and behavior in humans serve the same function in rodents, making them a suitable experimental population for studying environmental effects during the perinatal period [10].

Mood and anxiety-like phenotypes in rodents

Although the complex nature of mood disorders makes them almost impossible to fully model in rodents, specific features can be observed. It is beyond the scope of this chapter to present a detailed review of all behavioral assays used to assess anxiety- and depressive-like behaviors in rodents (see Table 9.1). However, multiple comprehensive reviews on the subject do exist (e.g., [33, 34]).

Prenatal exposures

Dr. David Barker is recognized for his work investigating the link between the prenatal environment and the diseases that occur in adulthood as a result of the prenatal environment. The Barker Hypothesis, also known as the Fetal Origin of Adult Disease Hypothesis [35], states that exposures to environmental challenges during early fetal life confer disease risk to the organism in adulthood. Environmental challenges include exposure to toxins, hormonal imbalances, maternal stress or depression, maternal substance abuse, infection, and nutritional deficiencies. Exposure to these environmental insults *in utero*, during critical periods of development, may act directly on the fetus or indirectly by disrupting the normal function of the placenta, resulting in decreased efficiency to support the fetus.

Evidence for the impact of uterine environment on the developing fetus and increased risk for neurodevelopmental disorders has been shown in case studies of children that experienced intrauterine growth restriction (IUGR) or were born small for gestation age (SGA). IUGR and SGA have been shown to heighten risk for cognitive deficits [36], stress-related disorders [37], learning disabilities [38], and attention deficithyperactivity disorder (ADHD) [39]. In this section, we explore animal

Table 9.1 Commonly Used Animal Models of Anxiety- and Depressive-Like Phenotypes

Factor	Human Correlate	Animal Model	Reference
Depression	Despair	Forced swim test	Porsolt et al. [11]
		Tail suspension test	Steru et al. [12]
		Learned helplessness	Seligman and Beagley [13]
	Anhedonia	Saccharine or sucrose consumption, intracranial self-stimulation	Berlin et al. [14], Le Pen et al. [15]
	Social withdrawal	Social interaction	Sams-Dodd [16]
		Resident intruder	Ellenbroek and Cools [17]
		Nest building	Belforte et al. [18]
		Home cage social behavior	Torres et al. [19]
	Avolition (lack of motivation)	Progressive ratio	Wiley and Compton [20]
Anxiety		Open field	Prut and Belzung [21]
		Elevated plus maze	Rodgers and Johnson [22]
		Light/dark	Crawley [23]
		Marble burying	Njung'e and Handley [24]
		Hole board	Gardner and Piper [25]
	Anticipatory anxiety	Stress-induced hypothermia	Borsini et al. [26]
Stress	Stressful life event	Restraint (acute or chronic)	Herrenkohl and Whitney [27]
		Mild stress of a novel environment	Piazza et al. [28]
		Chronic mild stress	Willner et al. [29]
		Chronic unpredictable stress	Katz [30]
	Social Stress	Chronic social defeat	Kudryavtseva et al. [31]
Self-care		Grooming, body weight, coat condition, general health, barbering, etc.	Audet et al. [32]

models that can be used to mimic environmental perturbations during gestation. We will also discuss epigenetic changes resulting from these environmental exposures that can lead to behavioral and gene expression changes in adulthood.

Maternal mood and stress

Numerous studies in humans have shown that maternal stress and depression have adverse effects on the offspring [40–43] and can lead to increased risk for development of psychiatric disorders in adulthood [44]. Evidence for epigenetic changes in response to maternal stress and depression comes from rodent studies. In the 1950s, Thompson [45] introduced the original model of rodent maternal stress. Rats were trained to associate a conditioned stimulus (CS) with an unconditioned stimulus (US) and then to escape from the US using a shuttle box apparatus. The rats were then mated and, during the last week of gestation, re-exposed to the conditioned stimulus with the escape route blocked, creating a stressful situation. Pups were cross-fostered to allow for separation of prenatal and postnatal stress effects. Pups from mothers exposed to stress exhibited increased

anxiety-, depressive-, and stress-related behaviors [45–47]. Mothers were exposed to stress before and during gestation but it is now more common to measure these effects in separate experimental groups.

Although many laboratories still use the US–CS shuttle box method, other commonly used stressors include restraint [27], overcrowding [48], and a combination of stressors in the chronic variable (or unpredictable) stress paradigm [49]. Variations of these methods include the gestational period at which the stressor is applied, the duration of the stressor, predictable compared with unpredictable timing of stressors, acute compared with chronic stress, and exposure to a combination of different stressors compared with repeated use of a single stressor.

The chronic variable stress (CVS) paradigm can include a variety of stressors such as forced swim, restraint, elevated plus maze, bright light, novel objects, novel noise, frequent cage changes, saturated bedding, exposure to predator odors, water deprivation, cage overcrowding, and social isolation. Male mice and rats exposed to CVS *in utero* show increased depressive- and anxiety-related behaviors, including increased immobility in the forced swim and tail suspension tests, increased anhedonia indicated by reduced sucrose intake [49,50], reduced exploratory behavior [51,52], decreased social interaction [52], and increased corticosterone (CORT) levels in response to stress [49,53–55]. There is evidence that the timing of exposure to stress during gestation does matter, with early pregnancy being the most sensitive in male mice [49].

Male mice exposed to CVS in early gestation exhibit an increase in CRF and a decrease in glucocorticoid receptor (GR) gene (*Nr3c1*) expression in adulthood [49]. Investigation of possible epigenetic mechanisms that would account for altered gene expression revealed a decrease in DNA methylation in the *Crh* promoter and an increase in methylation of the *Nr3c1* exon 17 promoter region in the hypothalamus. Interestingly, examination of placental gene expression showed a sex-dependent difference in levels of the DNA methyltransferase 1 gene (*Dnmt1*). Control females had significantly higher levels than control males [49]—a difference that could account for sex differences seen in behavior.

Exposure to toxins

Bisphenol-A (BPA)

Bisphenol-A (BPA) is a synthetic xenoestrogen that can mimic the effects of estrogen in the body. Due to its ability to produce epoxy resins, BPA is frequently used as a synthetic monomer in the manufacture of polycarbonate plastic, polystyrene resins, and dental sealants. Roughly 6.5 billion pounds of BPA are produced each year, and it is the major estrogenic compound that contaminates our water supply [56]. Exposure to BPA has been shown to affect both reproductive tissues and the brain [57,58], and work has focused on investigating the effects of BPA exposure during gestation.

Rodent studies have found that prenatal exposure to BPA results in impairments in learning and memory in the Morris water maze and the step-down passive avoidance task [59], decreased time and entries into the open arms of an elevated plus maze [60,61], decreased exploratory activity, and increased CORT levels after a mild stressor [62]. Interestingly, sex differences that are normally observed in anxiety-related assays were not observed after BPA treatment, indicating that BPA might eliminate sexual dimorphism for some of these behaviors [63].

Prenatal BPA exposure also resulted in decreased estrogen receptor β and decreased expression of the *N*-methyl-D-aspartate (NMDA) glutamate receptor in the hippocampus [59]. The epigenetic mechanisms of BPA action are poorly understood, and at this point it is highly speculative to assume that these mechanisms are independent of the estrogenic activity of BPA. It is known that *in utero*

exposure to BPA induces widespread changes in DNA methylation in the brain, but the mechanism and specific genes affected have yet to be determined [58].

Methylmercury

Methylmercury (MeHg) is a well-known neurotoxic compound. The main source of human exposure is contaminated seafood [64]. Case studies of human populations in Japan [65] and Iraq [66] that were exposed to MeHg poisoning have provided convincing evidence for the susceptibility of the developing brain in the prenatal period to MeHg exposure and resultant negative consequences on offspring in later life. These studies have led to investigation of the impact of lower levels of MeHg exposure on human neurodevelopment [67]. Two case studies of seafood-eating communities in the Faroe Islands [68] and New Zealand [69] found dose-related effects on increased risk for development of several neuropsychiatric disorders.

Male mice exposed to MeHg during the perinatal period exhibit behavioral changes in adulthood such as decreased exploratory activity, reduced working memory performance, and depressive-like behaviors, including increased immobility in the forced swim test [70,71]. Interestingly, forced swim immobility behavior is reversible with chronic antidepressant treatment [71]. These behavioral phenotypes are thought to be due to decreased BDNF in the hippocampus, which can be reversed with antidepressant treatment. Investigation of possible epigenetic mechanisms revealed DNA hypermethylation, increased histone H3K27 trimethylation, and decreased H3 acetylation within the IV promoter of the *BdnF* gene in the hippocampus. These perinatal MeHg-mediated effects result in long-lasting repression of the chromatin region at the *BdnF* promoter and, therefore, decreased levels of BDNF mRNA [71].

Nutritional deficiencies

The Dutch Hunger Winter or Dutch famine (1944–1945) and the Great Chinese Famine (1959–1961) are two of the most studied "natural" experiments of developmental nutritional deficiency in humans. The Dutch Hunger Winter occurred in several cities in western Holland at the end of World War II as a result of a German blockade that cut off food and fuel shipments along with a particularly severe winter. People in these cities had been relatively well nourished but daily food rations fell to 800 calories or less during the height of the famine. Medical records were well documented before, during, and after the famine. These documents gave researchers a unique experimental population to study the effects of nutritional deficiencies during specific periods before and during gestation and in the postpartum period. The Dutch Hunger Winter cohort consisted of 2414 children born between November 1943 and February 1947 in the Wilhelmina Gasthuis hospital in Amsterdam. Any individual who received less than 1000 calories during any 13 weeks of pregnancy was considered to be nutritionally deficient during that gestational phase. The cohort was broken down by children exposed to nutritional deficiencies in early, mid, and late gestation. These children were followed throughout life and assessed at ages 50 and 58 [72–74]. The Great Chinese Famine of 1959–1961 affected all provinces of China, with Anhui being the most devastated. The famine was the result of political and economic policies, drought, and poor weather [75]. This population was also relatively well nourished prior to the famine and kept detailed medical records. These two experimental populations provided the framework to determine the effect of perinatal exposures to nutritional deficits at critical periods of development and to follow offspring into adulthood to study the effects of gestational nutrient deprivation and risk for adult disease. Findings from the Dutch Hunger Winter and the Great

Chinese Famine cohorts indicate that offspring exposed to famine *in utero* have an increased risk for schizophrenia and affective disorders that is dependent on gender and timing of exposure [75–79]. There is also evidence of altered genome-wide and gene-specific DNA methylation patterns in adults who were exposed to famine in gestation during the Dutch famine [80–82].The findings from these studies provided evidence that maternal nutrition before and during pregnancy can affect disease susceptibility of offspring in adulthood [72–74].

Various perinatal nutritional deficiencies have been examined in rodent models. Mice exposed to prenatal protein deficiency exhibit altered behavior in models of schizophrenia including increased stereotypic response to apomorphine, increased locomotor response to amphetamine [83], and decreased prepulse inhibition of the startle reflex [84]. Interestingly these effects were both sex and age specific [83,84]. Mice exposed to protein deficiencies during the perinatal period exhibit decreased sucrose intake, hyperactivity, and exaggerated locomotor response to psychostimulants [85]. Developmental vitamin D deficiency results in enhanced sensitivity to psychostimulants in rats [86] and increased exploratory behavior and spontaneous hyperlocomotion in mice [87]. Rats exposed to gestational choline deficiency show increased anxiety-like behaviors in the elevated plus maze and increased learning ability in an assay for sensitivity to reinforcement [88].

Changes in methylation in response to various perinatal dietary deficiencies have also been observed in animal models. Perinatal protein deficiency in mice results in decreased methylation of the promoter region of cyclin-dependent kinase inhibitor 1c (*Cdkn1c*), leading to dysregulation of dopaminergic neuron development [85]. *In utero* protein deficiency in rats increased GR expression due to decreased DNA methylation of its promoter [89]. Vitamin D deficiency during gestation disrupts expression of genes involved in the developing dopaminergic system [86,90]. Gestational exposure to choline deficiency results in global DNA hypomethylation in the ventricular and subventricular zone of the hippocampus and hypomethylation of the promoter of the *Cdkn3* gene, a gene involved in cell-cycle regulation [91]. Choline deficiency during gestation also affects histone methylation patterns in rat frontal cortex by downregulating histone methyltransferase G9a (also known as *Kmt1c*) and Suv39h1 (also known as *Kmt1a*) [92]. Gestational choline supplementation has been shown to upregulate H3K9me2, a tag of transcriptionally repressed chromatin [92]. Collectively, these findings indicate that altering access to methyl donors during gestation can modify DNA and histone methylation in animal models.

Postnatal exposures

Brain development continues in the postnatal period and into adolescence. Therefore, the effects of environmental exposures during this time period must also be considered. Among the most studied environmental factors during the postnatal period are maternal care, bonding, and abuse and neglect of the offspring. In this section, we discuss how maternal interaction during the postnatal period is measured in animal models and how it affects adult behavior and gene expression.

Maternal care and bonding

Studies in humans have shown that unresponsive or inconsistent maternal care can result in insecure mother–infant attachment, which can increase the risk for psychiatric disorders in the offspring [93].

One aspect of maternal care in particular is tactile stimulation, or gentle touching and stroking of the infant by the mother. Tactile stimulation has effects on pain sensitivity and on the growth and development of the infant, and it leads to increased maternal responsiveness and infant attachment [94].

In rodents, maternal care and bonding can also be measured by the amount of maternal tactile stimulation, licking and grooming (LG), and arched back nursing (ABN) performed by the mother for her offspring. Maternal LG regulates endocrine systems that influence metabolism and somatic growth in pups. The frequency of LG has been shown to be consistent across litters in female rats and is considered to be a reliable measure of maternal care [95]. Offspring from high LG mothers display reduced ACTH and CORT responses to stress and decreased anxiety behaviors [96–98]. Conversely, offspring of low LG mothers have increased endocrine responses to stress and increased anxiety behaviors. Interestingly, differences in maternal behavior can also be transmitted across generations [97].

The ability of maternal tactile stimulation to alter the stress response and anxiety behavior in offspring is thought to be due to differential regulation of GRs in the hippocampus. Postnatal exposure to low LG reduced *Nr3c1* expression, and high LG exposure increased *Nr3c1* expression in the hippocampus in rats. Meaney and colleagues have explored the epigenetic mechanisms responsible for this bidirectional regulation of the *GR* gene. Maternal LG increases triiodothyronine (T3), which acts to increase 5-HT activity in the hippocampus and leads to an increase in nerve growth factor inducible A (*NGFIA*, also known as *Egr1*) gene expression. *Egr1* associates with the *Nr3c1* exon 17 promoter, causing an alteration in methylation [95–100]. Interestingly, this effect is also seen by artificial tactile stimulation of the pup with a brush [101] and can be reversed by cross-fostering [97,102,103]. Another proposed mechanism by which maternal care can alter the stress response is by altering CRF expression in key brain regions that regulate stress. CRF expression dysregulation has been shown to be the result of altered gamma aminobutyric acid A ($GABA_A$) receptor mRNA subunit expression caused by maternal care in early life. Pups of high LG–ABN mothers showed differing subunit composition than pups reared by low LG–ABN mothers, and this effect was reversed by cross-fostering. This finding suggests that maternal care can permanently alter $GABA_A$ receptor composition to alter the inhibition of CRF in the brain resulting in dysregulation of the stress response [102].

Inbred strains of mice have also been used to examine the effects of natural variation in maternal care. C57BL/6 (B6) and BALB/c (BALB) mice show significant differences in mothering behavior [104–107]. B6 mice are considered good mothers as they show high amounts of LG–ABN, and BALB are considered poor mothers as they exhibit less LG–ABN, spend less time on the nest, and have longer latencies to retrieve pups that have left the nest [104–108]. Interestingly, these two strains also differ in anxiety and stress behaviors. B6 mice are less anxious in multiple animal models of anxiety as compared with BALB mice [107–111] and also show significantly lower CORT and ACTH levels in response to stress as well as basal CRF levels [105–107,112]. Differences in anxiety, stress response, and mothering behavior have led to the use of these two strains to look at the effects of genetics, mothering behavior, and their interaction on behavioral differences [113]. The extent to which mothering behavior influences adult behaviors in these strains has been studied in cross-fostering experiments. In rodents, cross-fostering involves removal of pups from their biological mother at birth and placing them with a foster mother, and allows for examination of the effects of genes and the environment separately. In B6 and BALB mice, studies utilizing cross-fostering have yielded differing results. For B6 mice fostered by a BALB mother, some studies found an increase in anxiety behavior and CORT levels in response to stress [107,110], while other studies observed

no change [105]. BALB mice fostered to B6 mothers have been reported to show decreased anxiety in some behavioral tests such as the elevated plus maze [107] and Morris water maze [105], but no change in the open field [107]. The mixed findings from these studies indicate that the effects of maternal care are not sufficient to explain the behavioral differences between these strains, and variations in maternal care result in different outcomes depending upon which behavioral test is employed (even tests within the same domain). Very few studies have examined the effects of maternal separation in either strain, and within those studies there have been mixed results on the offspring [108,114,115]. Further studies to examine neuroendocrine and behavioral changes in B6 and BALB dams during gestation and the postpartum period are necessary to begin disentangling genetic and environmental components that lead to behavioral differences in the offspring.

Maternal abuse and neglect

Because maternal care and bonding in the postnatal period have been shown to have epigenetic effects and can result in behavioral changes in the offspring, it is worth considering extreme adverse forms of maternal care, such as abuse or neglect. Childhood abuse is a significant predictor of increased risk for development of a psychiatric disorder in adulthood [116,117]. Postmortem brain tissue from suicide victims with a history of childhood abuse showed reduced GR gene expression compared with tissue from victims without a history of abuse and non-suicide controls. Reduced GR gene expression was due to a hypermethylation of the GR promoter [118].

Maternal separation is the most common animal model of neglect. It involves repeated removal of the pups from the mother during the early postnatal period. The duration of separation is an important consideration when using this method. Short periods of maternal separation (3–15 minutes) result in a stress-resistant phenotype in the offspring, but longer separation durations (3–6 hours) result in a stress-susceptible phenotype [119].

Stress resistance in pups separated from the dam seems counterintuitive; however, in comparison to dams whose pups are removed for longer durations, dams of pups taken away for short periods of time show increased LG–ABN when pups are returned to the cage [120]. Therefore, it is thought that increased maternal care after short-term separation actually promotes active coping strategies in the pups, resulting in decreased anxiety and stress behaviors [119,121]. These effects may be due to decreased CRF in the paraventricular nucleus (PVN) of the hypothalamus along with reduced glutamatergic input to these neurons and elevated neuron-restrictive silencer factor (NRSF), which negatively regulates CRF expression [121]. Animals exposed to short-term maternal separation also exhibit increased GR expression in the hippocampus and decreased CORT levels in response to stress [120,122,123].

Pups exposed to longer maternal separation exhibit increased stress-induced CORT levels and impaired passive coping and memory [124,125]. This is thought to be a result of increased *Crf* gene expression and decreases in GR and $GABA_A$ receptor subunit gene expression [96,97,126]. Elevated levels of NRSF, which acts to suppress *Crh* gene transcription, may also be responsible for these behavioral outcomes [124]. In addition, Murgatroyd et al. [124] observed the loss of *Mecp2* regulation in these animals, leading to hypomethylation of the *Avp* promoter and increased expression of AVP in the PVN, an important component of the HPA axis. Interestingly, another study found that one long period of maternal separation also resulted in increased CORT levels but decreased AVP expression in the hippocampus [125]. This decrease in *Avp* expression was due to hypermethylation of its gene promoter in this brain region. Both of these studies suggest that regulation of

AVP expression may be one mechanism by which long periods of maternal separation in early life can result in lifelong changes in the stress response and that these alterations can be brain region specific.

Maternal neglect can by modeled in rodents by reducing nesting materials in the cage of a lactating female during the postnatal period. This paradigm has been shown to significantly increase aggressive behaviors of the mother toward her pups including rough handling, active avoidance, aggressive grooming, transporting pups by a single limb, dropping pups, and decreased nurturing behaviors such as LG [127,128]. Audible and ultrasonic distress calls from pups are measured to assess the pup's response to the caregiver [129].

Animals exposed to maternal neglect in the postnatal period exhibit deficits in learning and memory that may be due to increased CRF in the hippocampus leading to over activation of the CRF receptor [130]. These animals also exhibit dysregulation of the stress response as indicated by increased basal CORT levels and sustained elevated CORT levels after exposure to a stressful event [131]. Exposure to this form of maternal neglect also results in reduced BDNF expression in the prefrontal cortex (PFC) due to increased DNA methylation of the promoter region of *Bdnf* [132]. However, it is of interest that these changes in BDNF were different between males and females across the lifespan [129]. Further investigation of genes involved in methylation in the PFC revealed a decrease in mRNA levels of *Dnmt1*, *Dnmt3a*, *Mecp2*, and Growth arrest and DNA-damage-inducible 45 beta (*Gadd45b*) in males and a decrease in only *Gadd45b* in females [133].

Adulthood exposures

It has become more evident in recent years that epigenetic plasticity in adulthood is also a time window to consider when examining the factors leading to increased risk for development of neuropsychiatric disorders. Stressful life events that can occur during adulthood, such as divorce/separation, death of a loved one, severe illness or injury, assault, financial trouble, or serious trouble within one's social network, have all been shown to lead to the onset of an affective disorder [134,135]. A twin study investigating risk for developing depression in response to stressful life events found that increased risk was substantially affected by genetic predisposition [136]. Furthermore, there are reported sex differences in sensitivity to stressful life events. Men are more susceptible to developing depression after divorce/separation or work troubles and women are more vulnerable to problems within their social group, serious illness or injury, or the death of a loved one [135]. Dissecting out mechanisms that increase susceptibility in certain individuals to develop affective disorders after exposure to a stressful life event in adulthood is complicated by previous exposures earlier in life. Controlling for all possible environmental exposures that one may have encountered during his or her life that could lead to increased risk for neuropsychiatric disorders is almost an insurmountable challenge in humans. Animal models of stressful events in adulthood can be indispensable in this regard. Another environmental challenge that occurs in adulthood that has been linked to increased risk for development of affective disorders is naturally occurring changes in reproductive hormones. Dramatic changes in estrogen levels are seen in women during the perinatal and postpartum period and also during menopause. During this time, women are at an increased risk for development of depression and anxiety, which is thought to be the result of both genetic predisposition, hormonal changes and their interaction.

In the next section, we will focus on animal models of stress (with a specific emphasis on social stress) that have been shown to result in changes in behaviors that model psychiatric illness. We will also explore the role of normal hormonal changes that occur in women and increase risk for mood disorders.

Social stress

Social stressors have been shown to play a role in inducing depression and anxiety in humans, and behavioral models of social stress in rodents alter anxiety- and depressive-related behaviors [137]. Due to its high constructive, discriminative, and face validity, chronic social defeat is one of the most commonly utilized animal models to induce stress in adult rodents [137]. Chronic social defeat is a modified version of the resident–intruder paradigm in which a male rodent, the intruder, is placed in the home cage of a resident male that has been shown to display dominant and aggressive behavior for a period of 5–15 minutes daily for several weeks. A modification of this paradigm is the sensory contact assay, in which the intruder male is placed in the resident male's cage daily but is also subjected to continuous psychological stress by being housed in a compartment adjacent to the aggressive resident male that allows for constant sensory stimulation from the aggressor. Chronic social defeat paradigms model persistent psychological stress and differentiate between individuals that are susceptible or resilient to the effects of social stress [31].

Exposure to social defeat results in social avoidance, increased submissive behaviors, reduced sexual behavior, and reduced aggression in susceptible intruder males [138–141]. These males also show increased depressive-like behavior, including increased immobility and decreased latency to immobility in the forced swim test and reduced sucrose intake indicating anhedonia [141–143], in addition to anxiety-like behavior marked by decreased locomotion and exploratory activity in a novel environment and increased time in the closed arms of an elevated plus maze [139–142]. Males that undergo chronic social defeat also show an altered stress response indicated by increased CORT levels after exposure to a stressful event [142]. The chronic social defeat paradigm is used mainly in males, as female rodents do not display the same territorial aggression as males. However, it has been shown to work in females that are nursing, as they will display aggressive behaviors toward intruders [144].

Proposed epigenetic modifications resulting from chronic social defeat include decreased DNA methylation of the *Crh* promoter in the PVN of susceptible mice [145]. LaPlant et al. [141] also found that chronic social defeat resulted in increased levels of *Dnmt3a* in the nucleus accumbens (NAc) and that treatment with a DNMT inhibitor prevented the development of social avoidance seen after social defeat [141]. Tsankova et al. [146] reported decreased hippocampal expression of two *Bdnf* transcripts in susceptible mice, resulting from hypoacetylation of the *Bdnf* promoter. Furthermore, it has been shown that hyperacetylation of the *Bdnf* promoter in the hippocampus can block the effects of chronic social defeat. Another study from the same laboratory found that BDNF is increased in the NAc after chronic social defeat and that deletion of BDNF from the ventral tegmental area, which is a major source of BDNF to the NAc, or treatment with antidepressants results in a reversal of social avoidance behavior [138].

Stressful life events

Chronic restraint stress is commonly used in both male and female rodents. In this model, rodents are placed in a cylinder that prevents mobility for several hours every day for at least two weeks (typically

14 to 30 days). Restraint stress has been shown to increase CORT levels [147], anxiety-like behaviors in the open field and elevated plus maze and depressive-like behaviors in the forced swim test, and to decrease sucrose intake [147–150]. These effects are potentially due to increased glucocorticoids, which act to downregulate GR expression [147]. There are also conflicting reports about a possible role for BDNF [147,148,151]. It should be noted that animals often adapt to chronic restraint stress, and for this reason many laboratories have started using CVS (described above) in adult animals as a means of studying the effects of chronic stress on anxiety- and depressive-related behaviors.

Another form of stress used in adult animals is exposure to a novel environment. This stressor is effective in both males and females and is used as a predictive tool to differentiate between individuals that are resilient or susceptible to the effects of stress. In this paradigm, animals are briefly exposed to a new environment, such as an open field, and the amount of exploratory activity, total distance moved, or total activity is measured. Based on the degree of exploratory activity the animal can be classified as a high responder (HR) or low responder (LR) [28,152–154]. Classification of HR and LR to a novel environment has been shown to predict behavioral responses to other environmental challenges including social defeat and depressive- and anxiety-like behaviors [152–155]. The behavioral differences that exist between HR and LR rodents could be the result of differences in neuronal activation of specific brain regions in response to stress. A study by Kabbaj and Akil [152] found differences in c-*fos* mRNA, a marker of neuronal activation, after a 5-minute stressor with HR, showing low c-*fos* levels in CA1 of the hippocampus and high c-*fos* levels in the PFC, dorsal striatum, and PVN compared with LR. Another study found differences in the dopaminergic system between HR and LR animals, with HR rats showing an increase in behavioral response (activity) to DA infused into the NAc, less D_2 receptor-binding sites in the NAc and striatum, and increased D_1 receptor-binding in the NAc compared with LR rats [156]. A recent report investigated epigenetic differences between HR and LR animals and found differences in histone acetylation in the hippocampus measured both before and after a social defeat experience [154]. Much more work is needed to determine specific genetic polymorphisms and epigenetic alterations and how they alter anxiety and depressive-like behaviors.

Reproductive hormones

A women's risk for mood disorders increases during times of hormonal flux—at the onset of menses, during the perinatal and postpartum period, and at menopause [157], but not all women will develop a mood disorder during these sensitive periods. Increased vulnerability during times of extreme hormonal flux is the result of both genetic predisposition and hormonal changes, leading to the notion that epigenetic modifications might be an underlying mechanism. Animal models have been and continue to be useful for studying the role of genes and environment on vulnerability to mood disorders during times of hormonal flux.

Daily injections of estrogen and progesterone for 21 days followed by a sharp withdrawal of both hormones mimics pregnancy and the postpartum period in rodents [158]. Dams exposed to this paradigm display increased depressive-like behavior in the forced swim test during the postnatal period, underscoring its utility as a model of postpartum depression [158]. Interestingly, this phenotype can be reversed with estrogen treatment [158].

Another model of hormonal withdrawal is bilateral ovariectomy, which results in a sharp decline of circulating estrogen levels mimicking the menopausal period [159,160]. Studies have shown that

ovariectomy results in an increase in anxiety-like behavior in the elevated plus maze [159,161] and an increase in immobility in the forced swim test [159,160,162–165]. Estrogen replacement can also prevent the depressive and anxiety-like behavioral changes in ovariectomized animals [160,163]. The effects of estrogen depletion on behavior are thought to be modulated by the 5-HT system both directly through 5-HT receptors and indirectly through the nitric oxide pathway [162,163].

Results from animal studies of hormonal flux highlight the need to further investigate these models to identify potential underlying epigenetic modifications (e.g., DNA methylation, histone acetylation), specific gene promoters (e.g., 5-HT), and specific brain regions that are modulated by estrogen flux in adulthood. Additional research is also needed to investigate possible epigenetic modifications caused by changes in progesterone levels that are also fluctuating during the perinatal period. Progesterone is known to interact with the HPA axis along with estrogen to increase risk for development of depression [166,167].

Future directions

Sex differences in epigenetic modifications

Additional research is needed to further investigate sex-specific epigenetic changes in response to the environment. Within the human population, the lifetime prevalence rate for development of an affective disorder is twice as high in females compared with males [1]. However, in animal studies, males are more commonly used than females, and when females are included effects of sex are not always taken into account. Previous reports in rodents have shown sex-dependent differences in response to environmental stressors in the development of anxiety- and depressive-like phenotypes [49,50,129,149]. The literature seems to support the idea that males are more susceptible to prenatal and postnatal stress than females, while females are more vulnerable to stress in adulthood [168,169]. However, this could be due to the disproportionate amount of research done in males. In addition, more research is required to investigate sex-specific differences in epigenetic mechanisms in response to stress. For example, *Dnmt3a*, a key epigenetic target of stress, is hypothesized to play a role in regulating stress susceptibility in a sex-dependent manner [141].

New experimental populations

Much of the work on epigenetic responses to environmental factors was pioneered in rats, but inbred strains of mice are now more commonly utilized. The use of inbred mice as an experimental population offers several advantages: (1) hundreds of inbred strains exist, allowing for cross-strain comparisons and genetic mapping; (2) genetic background is fixed in inbred strains; (3) genomic resources, including full sequence data, are available for many strains; and (4) reference mouse populations such as inbred strains can be recapitulated at any time for biological replication and more targeted studies [170].

The advantages of using inbred strains of mice are especially important when investigating the interaction of environmental factors and genetics. Many of the studies discussed in this chapter were done in inbred mouse strains, but very few studies have taken full advantage of the usefulness of comparing across inbred strains. Although mice of one inbred strain are genetically identical, naturally occurring genetic and phenotypic variation exists among inbred strains of mice—both for baseline behavior and in epigenetic responses to environmental challenges. This phenotypic and genetic

variation has been used for decades to further our understanding of the genetic basis of human disease. In the past few years, advanced genetic and genomic tools have emerged for the mouse that can aid in the identification of specific genes that are altered by exposure to the environment. These include the emergence of databases that contain phenotype, gene expression, and single nucleotide polymorphism data across hundreds of inbred strains [171,172] and the emergence of new experimental populations of mice that have been optimized to study systems genetics [173]. For example, the Collaborative Cross (CC) is a large panel of recombinant inbred (RI) mouse strains created from breeding eight founder mouse strains (A/J, C57BL/6J, 129S1/SvlmJ, NOD/ItJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) that were selected to optimize genetic diversity specifically for use in complex trait analysis [173]. Inbred mouse strains allow for comparison of natural phenotypic and genetic variations, but it is thought that some of the phenotypic diversity is hidden among inbred strains that share common genetic variation. Use of the CC lines will amplify naturally occurring phenotypic and genetic diversity. Furthermore, CC lines can be bred together to form F_1 progeny (recombinant inbred intercrosses, or RIX). RIX mice also have reproducible genotypes and will be more representative of the heterogeneity found in the human population. Use of these mice will greatly enrich genetic analysis to allow for precisely mapped candidate regions. This allows one to fully elucidate the relationship among environmental factors, a specific genotype, and the resulting phenotypic outcome.

Measure gene-specific modifications

Overall, there is a need for precise measurement of gene-specific modifications *in vivo* to allow for assessment of the link between specific epigenetic changes and the resulting behavioral outcome. When a gene of interest has been identified, techniques are needed for assessing the exact function of the gene in its normal state and when it has been modified (e.g., by an environmental factor). Manipulation and measurement at the gene level has long been a challenge; however, new and emerging technologies have given way to what is sure to become an emergence of experiments done with precise control of genome regulation, including gene transcription and epigenetic modifications such as histone regulation. One such tool is the use of DNA-binding proteins such as transcriptional activator-like effectors (TALEs) and zinc finger proteins, which allow for the binding of specific effector domains to exert changes in gene transcription or epigenetic marks to a targeted gene [174]. Fluorescent proteins can also be fused to DNA-binding domains to illuminate chromosomal configuration [174]. Using this technology, Konermann et al. [175] engineered a system by which transcription of genes can be optically controlled by light-inducible transcriptional effectors. Briefly, TALEs are combined with light-sensitive proteins that, when activated by blue light, recruit the necessary effector proteins that can either activate or repress gene transcription, resulting in increased or decreased expression of that specific gene. Interestingly, the authors were also able to construct a variety of repressive histone effector domains, including histone deacetylases, methyltransferases, and acetyltransferase inhibitors, which when activated by blue light resulted in decreased mRNA levels of the target genes. This technology has utilized optogenetics to control gene transcription and a specific epigenetic modification, histone modifications, *in vivo* in mouse brain. Using viral vectors and/or transgenic mice, this system is able to target specific genes within either the entire brain or specific regions at specific time points throughout the lifespan. This will allow for researchers to precisely target a gene with great temporal and spatial location to investigate its role in normal and disease states, such as anxiety and depressive-like behaviors.

Conclusions

In this chapter, we examined animal models of environmental challenges that have been shown to alter behaviors that model aspects of anxiety and depression and cause epigenetic modifications and changes in gene expression. These challenges include maternal exposure to stress, toxins, and nutritional deficiencies in the prenatal period, maternal care in the postnatal period, and stressful life events and naturally occurring hormonal changes in adulthood. These environmental challenges have been shown to induce epigenetic modifications such as DNA methylation and histone acetylation to key genes that are proposed to be involved in the underlying pathology of mood disorders including *Crh*, *Nr3c1*, and *Bdnf* (see Figure 9.1 for a summary). This chapter does not consider environmental factors during the adolescent period. This period is one of drastic changes in the body and also of

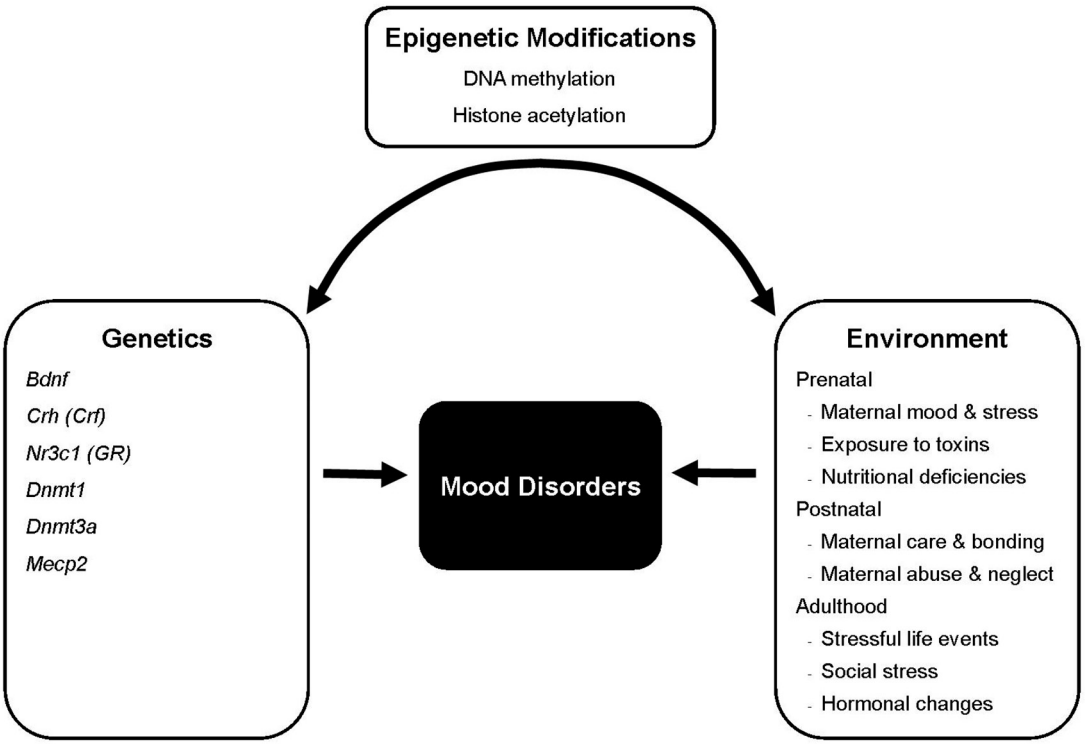

FIGURE 9.1

Genetic and environmental factors associated with behaviors that model mood disorders in animal models. Genetic predisposition, environmental exposures, and gene–environment interactions have all been shown to increase risk for developing psychiatric disease. Listed here are environmental factors that have been shown to result in changes in animal behaviors that are believed to model mood disorders. Genes listed are those that have been shown in animal models to be epigenetically regulated (by DNA methylation and histone acetylation) as a result of environmental exposures.

heightened stress; therefore, more research is needed to investigate the role of epigenetic patterns and risk for development of affective disorders during this sensitive period. The studies presented herein highlight the usefulness of animal models for examining the mechanisms involved in the development of complex phenotypes, specifically mood disorders and animal behaviors that model anxiety and depression. Although this field has seen major advances there are still many questions left to answer. The emergence of new experimental populations, such as CC lines and new techniques to measure gene-specific changes, will aid in developing an understanding of the role of the environment, genetics, and their interaction in the development of psychiatric disorders.

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Glossary

Affective disorder Mental disorder that results in persistent negative changes in mood. In this review, we use it as a synonym for mood disorders, including depression and anxiety.

Anhedonia Inability of an act or object that normally creates pleasure to elicit that response.

Collaborative Cross (CC) Recombinant inbred lines created by breeding eight founder strains of inbred mice (A/J, C57BL/6J, 129S1/SvlmJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) chosen to maximize genetic diversity.

Cross-fostering In rodents, this process involves the removal of pups from their biological mother at birth and placing them with a foster mother. This allows for the examination of maternal care environment and genetic background separately.

Environment In the context of this review, environment is used to refer to exposures that individuals encounter during their lifetime beginning *in utero*. We use this term in the broadest sense to include hormonal, social, stressful, toxicological, and nutritional events.

Gene promoter Region of DNA that initiates transcription of a gene.

Intrauterine growth restriction (IUGR) Inadequate growth of the fetus *in utero* that can result from maternal nutritional deficiencies or insufficient oxygen supply, among other causes.

Perinatal period The period including both gestation and the postpartum period.

Phenotype Observable characteristics or traits of an organism.

Postnatal (postpartum) period In humans this usually refers to the first year after birth and the first 3 weeks in rodents.

Prenatal period Time *in utero* or prior to birth.

Small for gestational age (SGA) Babies that are born smaller than normal for their gestational age, usually a body weight that is 10% lower than average for their age.

CHAPTER

5-Methycytosine and 5-Hydroxymethylcytosine in Psychiatric Epigenetics

10

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KEY CONCEPTS

- Alterations in the transcriptome and the epigenome have been demonstrated in multiple types of neurons and brain regions in autism spectrum disorder, as well as in psychosis. Major depressive disorder and suicide completers may also exhibit distinct and overlapping alterations in the epigenome.
- We propose that DNA modifications represent interconvertible epigenetic states that are context dependent and likely associated with early developmental life events including stress.
- There is widespread interest in understanding whether genome-wide methylation (methylomic) biomarkers present in blood are useful in predicting bipolar disorder with or without psychosis, schizophrenia, major depressive disorder, autism spectrum disorder, suicide, and neuroleptic medication responsiveness.

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- A key problem in examining methylation profiles in patients with psychoses is that antipsychotics such as olanzapine and clozapine have intrinsic DNA demethylation activity.
- Hydroxymethylation is a stable epigenetic mark that is recognized by a group of hydroxymethyl reader proteins, one of which is MBD3.

ABBREVIATIONS[1]

5-C 5-Cytosine
5-caC 5-Carboxylcytosine
5-fC 5-Formylcytosine
5-gmC 5-Glucosylmethylcytosine
5-hmC 5-Hydroxymethylcytosine
5-hmU 5-Hydroxymethyluridine
5-mC 5-Methylcytosine
AID/APOBEC Activation-induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
ASD Autism spectrum disorder
BDNF Brain-derived neurotrophic factor
BER Base excision repair
BGT beta-Glucosyltransferase
BLA Basolateral amygdala
BP Bipolar disorder
CEBPA CCAAT/enhancer-binding protein alpha
ChIP Chromatin immune precipitation
CMS Cytosine-5-methylenesulfonate
COMT Catechol-*O*-methyltransferase
CON Control
CpG Cytosine–phosphate–guanine
DMP Differentially methylated position
DMR Differentially methylated region
DNA DMase DNA demethylase
DNMT DNA methyltransferases
ESCs Embryonic stem cells
GABA γ-Aminobutyric acid
GAD1 Glutamic acid decarboxylase 1 (aka GAD67)
GADD45 Growth arrest and DNA damage-inducible 45
HAT Histone acetyltransferase
HDAC Histone deacetylase
HGP Human Genome Project
hMeDIP Hydroxymethylated MeDIP
HMT Histone methyltransferase
HP1 Heterochromatin protein 1
IL-11 Interleukin-11
MALDI-TOF MS Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry
MBD Methyl-CpG-binding domain
MDD Major depressive disorder
MeCP2 Methyl-CpG-binding domain protein 2
MeDIP Methylated DNA immunoprecipitation
NCBI National Center for Biotechnology Information
ncRNA Non-coding RNA
NGFI-1A Nerve growth factor-inducible protein 1A
NGS Next-generation sequencing
NIH National Institutes of Health
NLM National Library of Medicine
NPS Non-psychiatric subject
PCR Polymerase chain reaction
PSY Psychosis
RELN Reelin
RRBS-seq Reduced representation bisulfite sequencing
SAH *S*-adenosylhomocysteine
SAM *S*-adenosylmethionine
siRNA Small interfering RNA
SMUG1 Single-strand-selective uracil–DNA glycosylase 1
SNP Single-nucleotide polymorphism
SZ Schizophrenia
TAB-seq TET1-assisted bisulfite sequencing
TDG Thymine–DNA glycosylase
TET Ten–eleven translocation
TF Transcription factor
TS-DMRs Tissue-specific differentially methylated regions
TSS Transcription start site
UCSC University of California Santa Cruz
UDG Uracil–DNA glycosylase
UDP-glucose Uridine diphosphate glucose

[1] All gene symbols can be obtained by searching the HUGO Gene Nomenclature Committee web page at http://www.genenames.org/.

Introduction to the epigenome

The concept of the epigenetic landscape was coined nearly 60 years ago by Conrad H. Waddington to describe the process by which developmental decisions are made [1]. Waddington was both a geneticist and a developmental biologist, and the term epigenetics derives from the combination of "epigenesis"

with "genetics" [2]. Although the meaning of the term has changed over the years, it has come to refer to the interaction of the environment with the genome in the context of gene regulation and memory. At the level of individual cells, this refers to extracellular signals that impact gene expression in response to external signals that are self-perpetuating [3]. Many current epigenetic biologists think of the field of epigenetics as referring explicitly to those molecular processes that regulate interactions among histones, activators, repressors, and DNA. This has raised some concern among more classically trained chromatin and molecular biologists who understand epigenetics to mean specifically how extracellular or extraneuronal signaling impacts DNA and histone modifications to facilitate changes in gene expression. With reference to psychiatric research, epigenetics initially referred to the interaction of environmental factors with the genome [4]. The increased interest in epigenetic mechanisms grew out of a disillusion with conventional genetic linkage/association studies, which often failed to replicate in larger populations [5]. At the time, genetic and environmental factors were studied separately [5], although evidence of an interaction between epigenetics and the genome leading to phenotypic expression began to permeate the literature [6]. The epigenetic hypothesis is attractive to biological psychiatrists for many reasons and includes the possibility of explaining: (1) the discordance rates among monozygotic twins, (2) the later age of onset of certain psychiatric disorders, (3) gender differences, (4) parent-of-origin effects, and, last but not least, (5) the clinical heterogeneity observed upon presentation and the fluctuating progression of the disease [6].

We use epigenetics here to mean primarily the fundamental processes impacting DNA in response to extracellular cues. There are a large number of epigenetic marks that impact histones and other proteins associated with chromatin and that are involved in gene regulation. However, in the context of psychiatric disorders such as schizophrenia (SZ) and autism spectrum disorder (ASD), there is considerable evidence to support the idea that changes in DNA methylation occur in response to environmental cues related to either early-life stress or the response of the brain to stress incurred as a consequence of prenatal, postnatal, or early adolescent trauma [7]. A range of diverse conditions that may occur during pregnancy, including smoking and alcohol abuse, poor nutrition, labored birth, maternal infection, and fetal oxygen deprivation, activate the maternal stress system, leading to increased circulating glucocorticoids. Increased levels of cortisol correlate with an increased level of methylation of the glucocorticoid receptor gene *NR3C1* in the fetus during the first trimester [8]. Furthermore, maternal anxiety is also associated with increased methylation of cytosine–phosphate–guanine (CpG) located in the nerve growth factor-inducible protein 1A (NGFI-1A) binding sites in exon 1F of *NR3C1* in cord blood [8].

Postnatal maternal care of rats has been linked to methylation of specific CpG sites in the glucocorticoid receptor in the hippocampus that is correlated with levels of mRNA expression [9]. Maltreatment during childhood has also been linked to decreased glucocorticoid receptor expression in suicide completers with a history of abuse as compared with non-abused suicide completers [10]. Interestingly, childhood maltreatment is associated with increased promoter methylation at specific sites within *NR3C1* in the hippocampus. Methylation at these same sites in *NR3C1* is observed in non-abused suicide completers, suggesting that in some cases similar epigenetic marks can produce different outcomes with respect to transcription. Although the relevance of these so-called silent methylation marks is not currently well known, it suggests that early-life events can produce epigenetic marks in particular brain regions at specific sites that reside in the genome over long periods of time [11].

The DNA methylation/demethylation pathways are shown schematically in Figure 10.1A. Methylation of cytosines is catalyzed by one of a family of DNA methyltransferases (DNMTs) by the addition of a methyl moiety from *S*-adenosylmethionine (SAM) to the 5 position of the cytosine ring producing

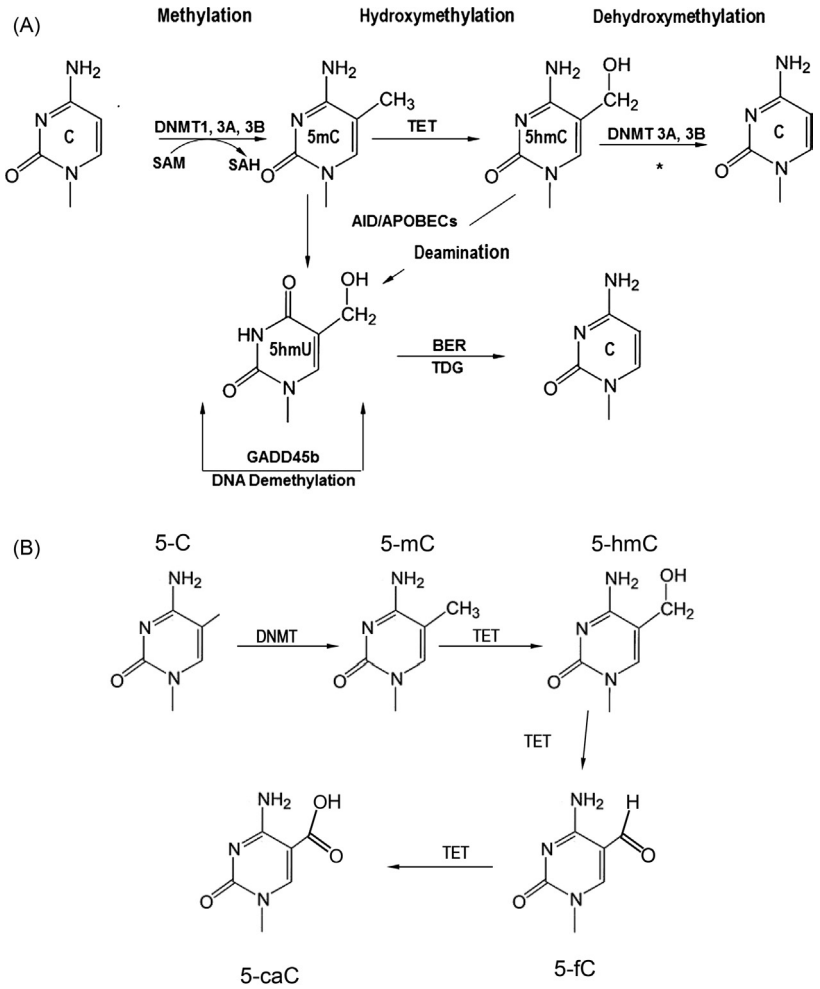

FIGURE 10.1

The dynamics of methylation–hydroxymethylation in mammals. (A) Sequential conversion of the cytosine base through a series of stable intermediates. The first step, catalyzed by DNMTs, adds a methyl group from the methyl donor SAM to the 5 position of the cytosine base. TET hydroxylates the methyl group forming 5-hmC*. At least one report suggests that 5-hmC can be directly demethylated by the activity of DNMT3A or DNMT3B as DNA dehydroxymethylases [149]. Alternatively, the next step involves an oxidative deamination of 5-hmC to produce 5-hydroxymethyluridine (5-hmU). However, the AID/APOBEC family of deaminases have little or no deamination activity [14]. This suggests that 5mC may be the preferred substrate for the AID/APOBEC enzymes or that 5-hmC may be directly dehydroxymethylated to form 5-hmU or 5-C, respectively. The intermediates are subsequently processed by the uracil–DNA glycosylase (UDG) family that includes thymine–DNA glycosylase (TDG) and single-strand-selective uracil–DNA glycosylase 1 (SMUG1). These latter steps are collectively part of the base excision repair (BER) glycosylases. TET oxidizes 5-hmC further to produce additional stable intermediates including 5-fC and 5-caC [150]. GADD45β is an activity-induced neuronal immediate early gene that facilitates active DNA demethylation [151]. This figure is modified from the original [13]. (B) Structures of the various modified cytosines discussed in this chapter. TET has the capacity to hydroxylate 5-mC and also subsequently forms 5-fC and 5-caC.

5-methylcytosine (5-mC). 5-Hydroxymethylcytosine (5-hmC) is formed by the hydroxylation of 5-mC which is catalyzed by one of three ten–eleven translocation methylcytosine dioxygenase enzymes (TET1–3). TET enzymes are 2-oxoglutarate- and Fe(II)-dependent enzymes that are also known as the TET methylcytosine dioxygenases (or TET hydroxylases). Subsequent steps in this pathway facilitate the conversion of 5-hmC to additional intermediates in the process of DNA demethylation [12–14]. The amount of 5-hmC varies across tissues, and in the brain can be as high as 40% of total CpGs in Purkinje neurons of the adult cerebellum [15]. 5-hmC can be further oxidized by TET to form 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC). The structures of each of these intermediates (5-mC, 5-hmC, 5-fC, and 5-caC) are shown in Figure 10.1B. The formation of 5-hmC is a stable but intermediate step in the demethylation process. Although 5-fC and 5-caC are present at much lower levels, they accumulate in the brain and each has a finite half-life. By far the most studied epigenetic DNA modification with respect to psychiatry is 5-mC, and we first provide an overview of DNA methylation and next highlight the role of DNA methylation in psychiatric disorders. It seems likely that only a subset of 5-mC residues become 5-hmC and that only a small fraction of the total CpG methylome is targeted by TET proteins. We conclude this chapter by providing a review of current findings in the literature regarding 5-hmC in psychiatric disorders.

CpG islands and DNA methylation

The methylation of cytosine at its 5 position was the first "epigenetic" modification to be described and studied in detail primarily because of its role in modifying the expression of tumor suppressor genes. The evolutionary origins of DNA methylation are unclear although it occurs in species as diverse as humans, mouse, *Arabidopsis thaliana*, and *Neurospora crassa* [16]. Interestingly, the plant methylome shares several similarities to the mammalian methylome, including the methylation of transposable elements and the lack of methylation proximal to promoter regions of actively transcribed genes [17]. Among the multiple eukaryotes that show only low levels of DNA methylation are *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Drosophila melanogaster*, and *Caenorhabditis elegans* [17]. In eukaryotes, cytosine methylation represses transcription and at the same time is present in gene bodies of actively transcribed genes. It appears that methylation plays different roles in different organisms although there is considerable conservation in the gene body regions of plants and animals [16]. The functions of DNA methylation are diverse and include transcriptional repression, X chromosome inactivation, imprinting, and the suppression of parasitic DNA sequences [18]. It was previously thought that in both eukaryotes and prokaryotes cytosine methylation acts as a fail-safe mechanism to silence the expression of foreign DNA such as transposable elements, proviruses, and other sequences [19].

Mammalian DNA methylation was thought to occur primarily on the cytosine of CpG dinucleotides. However, more recent genome-wide analyses show that extensive amounts of methylation also occur at CpH dinucleotides, where H = A, C, or T [20,21]. The idea that CpG islands are asymmetrically distributed in genes was suggested some 40 years ago [22]. Comprehensive analyses of CpG islands show that they are regions of DNA with a high G/C content and a high concentration of CpG dinucleotides relative to what might be expected in the bulk genome by chance [23]. CpG islands are features of large numbers of sequences of vertebrate genes that were initially associated with the 5′ flanking regions of housekeeping genes and many tissue-specific genes. Promoter proximal CpG islands exist in nearly 60% of all genes [24,25]. CpG-rich islands are unique and are largely unmethylated, and CpG island-containing promoters and enhancers are generally undermethylated (hypomethylated) consistent with a negative role for methylation in transcription [26]. The human *RELN* gene is proximal to a

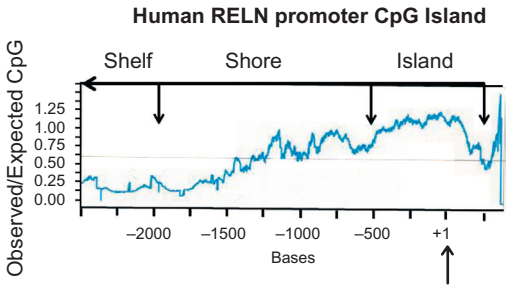

FIGURE 10.2

Schematic representation of the human RELN promoter proximal CpG island. The TSS is shown by the arrow at +1. The line represents the CpG density distribution across ~2.5 kb of 5′ flanking region and ~300 bp of 3′ sequence flanking the start site. The CpG island shore, as previously defined [27], extends both upstream (termed the north shore) and downstream (south shore). Further from the center of the CpG island reside the so-called CpG island north and south shelves. This figure is modified from the original [152].

CpG island which is often represented as a ratio of observed CpGs over expected (i.e., by chance) CpGs along the length of the gene (see Figure 10.2). The island extends from −550 bp upstream of the transcription start site (TSS) to about +350 bp downstream. The CpG island shore (also see below) extends another 2 kb upstream to 2 kb downstream of the center of the island [27].

Early *in vitro* experiments [28] showed that hypermethylation at promoter CpG islands decreases transcription. It was also shown early on that hypermethylation of tumor suppressor genes silences their expression and contributes to alterations in the cell cycle [18]. The effect of DNA methylation on gene expression in the brain has been extensively studied [29]. Two mechanisms are often invoked to explain methylation-mediated gene repression or silencing. The first is that methylation prevents the binding of sequence-specific transcription factors to their cognate recognition sites [30]. A second mechanism is based on the premise that methyl-CpG-binding (MBD) proteins including methyl-CpG-binding domain protein 2 (MeCP2) and MBD2 bind to the methylated DNA and facilitate a repressed state or closed conformational state [31]. Results of years of active research show that both possibilities most likely have merit; that is, there are cases where methylation within specific transcription binding sites interferes with binding, and it is also true that hypermethylated DNA domains exist in a compact/closed chromatin state. Current thinking suggests that closed chromatin is characterized by: (1) hypermethylated DNA, (2) the presence of MBD proteins, (3) deacetylated and methylated histones, and (4) additional repressor proteins. In contrast, transcriptionally active chromatin is more open with acetylated histones, demethylated promoter DNA, and the presence of bound transcription factors (Figure 10.3). The intermediate state shown represents a subclass of promoters that are poised for transcription and can be activated by, for example, histone deacetylase inhibitors (Figure 10.3).

DNA methylating enzymes

DNA methylation is carried out by a family of DNMTs that include DNMT1, 3A, 3B, and 3L [32]. DNMT3L is enzymatically inactive but shares sequence homology with both DNMT3A and

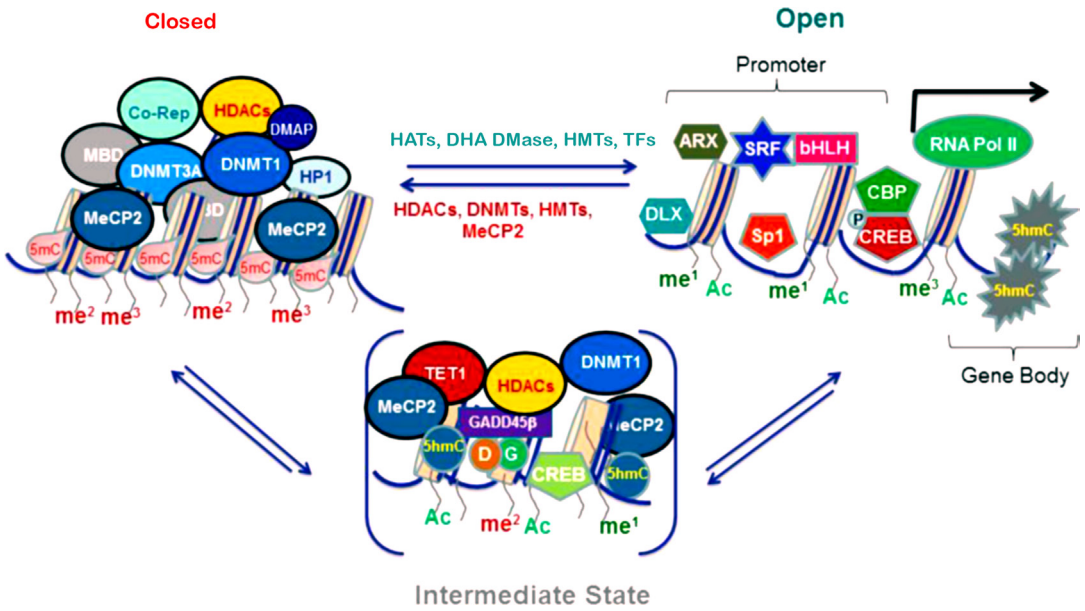

FIGURE 10.3

Representation of the transitions between closed chromatin and open chromatin. Closed chromatin is generally inactive transcriptionally and is characterized by the lack of DNase hypersensitive sites, a high degree of methylation, the binding of repressor proteins, and compact nucleosomes with little acetylated histones. In contrast, open chromatin is transcriptionally permissive and characterized by the binding of cell-specific transcription factors (TFs and co-activators) and the appearance of DNAseI hypersensitive sites due to the more open nature of the chromatin. This figure is modified from the original [13].

3B [33,34] and has recently been shown to antagonize DNA methylation at bivalent promoters [35]. SAM is the methyl donor used to transfer the one carbon methyl group by DNMTs producing 5-methylcytosine (5mC) and *S*-adenosylhomocysteine (SAH) in the process. In the nervous system, DNMT1 and 3A are likely to be major players in terms of postmitotic neuronal function [36]. Although DNMT3A is predominant in postmitotic neurons, there is increasing evidence that DNMT3B plays a secondary role in methylating DNA in response to extracellular cues in adults. The amino terminal half of DNMT1 contains a number of domains that target this activity to the nucleus (nuclear localization signals) and facilitate its interactions with numerous additional factors [37]. In most dividing (mitotically active) cells, DNMTs 3A and 3B function as *de novo* methyltransferases whereas the main role of DNMT1 is to maintain methylation patterns following DNA replication [38]. DNMT1 knockdown experiments using specific siRNAs demonstrate that loss of DNMT1 does not strictly alter maintenance DNA methylation patterns. In fact, it is becoming increasingly clear that both DNMT3A and 3B contribute to maintenance methylation and that DNMT1 may be required for *de novo* methylation [34,39]. DNMT3A and DNMT1 also cooperate functionally during *de novo* methylation, implying that both enzymes work in concert [40]. During cell division, DNMT3A appears to act to initiate *de novo* methylation, and the newly created methyl groups (hemi-methylation) activate DNMT1 to methylate the

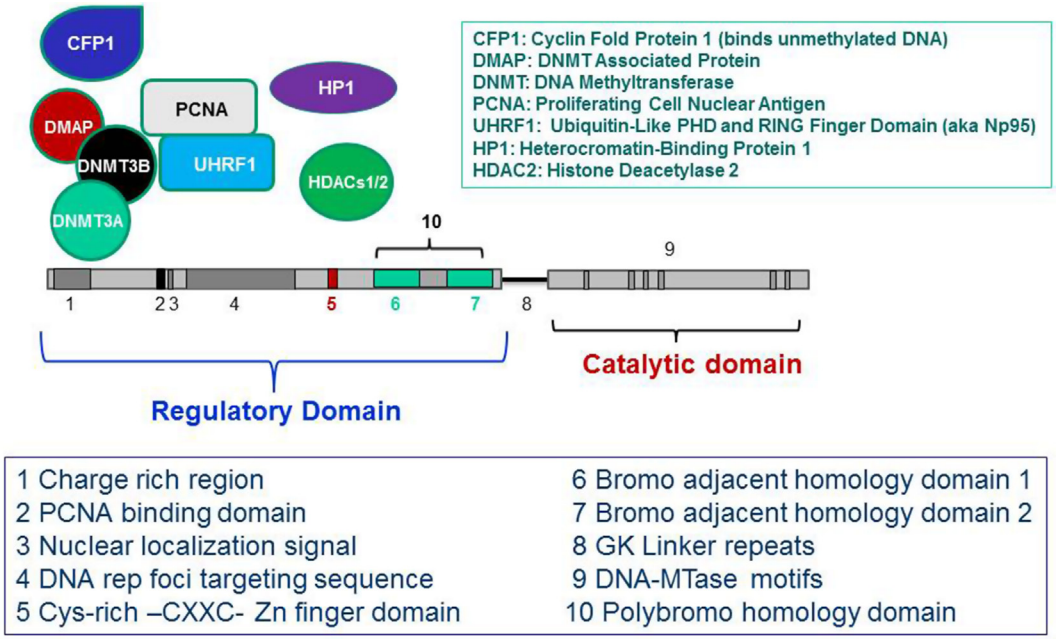

FIGURE 10.4

Domain organization of DNMT1. DNMT1 is linearly divided into a regulatory domain (amino terminus) and catalytic domains (carboxy terminus). Some of the interacting regions are numbered along with the name of the corresponding interacting protein. Key regulatory proteins are shown on top along with the associated names of these proteins [41,153]. The catalytic domains contain target recognition regions that have been shown to bind to RNA, as well as to DNA [154]. Interestingly, recent studies show that DNMT1 also binds with higher affinity to RNA molecules [147].

opposing strand. A key question regarding the functional roles of the DNMTs in neurons is whether they participate in gene repression beyond establishing DNA methylation patterns. Second, given the pleiotropic actions of DNMT1, are any of these functions specific to neurons and how are methylation profiles established in distinct brain regions?

As alluded to in the previous paragraph, DNMTs are comprised of flexible protein loops connecting various regulatory and catalytic domains that carry out distinct functions [32–33,41]. Recent studies show that the large amino-terminal region of DNMT1 contains a number of functional protein–protein and protein–DNA interacting domains (see Figure 10.4). The multidomain N-terminal region and the abundant protein interaction motifs suggest potential non-catalytic functions for DNMT1 [41–43]. A recent study showed that a DNMT1 mutant lacking catalytic activity but maintaining its DNA recognition domain still acts to repress the expression of a large set of target genes in a colon cancer cell line [44]. The role of DNMT1 in postmitotic neurons is still not entirely clear, although considerable evidence suggests that it binds to DNA acting as a repressor. In fact, the previous study referred to suggests a negative, *trans*-activating role for DNMT1 in most cells, including neurons. A recent genome-wide mapping study showed that each of the DNMTs binds to DNA and that the

presence of at least two DNMTs is strongly associated with genes targeted for DNA hypermethylation [45]. By comparing undifferentiated and retinoic acid-differentiated cell lines, the authors were able to demonstrate that DNMT3A is more enriched at heterochromatin following differentiation, while DNMT3B remained mostly associated with gene bodies after differentiation [45]. A comparison of DNMT1 binding before and after *in vitro* differentiation showed that this binding is coupled primarily to promoter regions of the differentiated cells [45]. Finally, it was noted that 5mC is not strictly linked to transcriptional repression as previously thought. That is, DNA methylation associated with transcriptional start sites correlates with repression whereas gene body methylation is positively associated with expression [46].

DNMT1 overexpression in psychosis

DNMT1 is of particular interest, as the levels of this enzyme are elevated in postmortem cortex and hippocampus of SZ patients compared with bipolar disorder patients with psychosis (BP+) and major depression subjects. Layer-specific increases in DNMT1 mRNA were first shown by *in situ* hybridization studies which also showed parallel decreases in the levels of glutamic acid decarboxylase 1 GAD1 (GAD67) and Reelin (RELN) mRNAs [47]. These data were replicated in a second cohort of subjects using both *in situ* hybridization and immunohistochemistry [48]. As was shown previously, DNMT1 mRNA and protein were increased in cortical layers I, II, and IV of BA9 of SZ and BP+ subjects. Additional studies have shown that in human [49] and mouse [50] brain, DNMT1 colocalizes with RELN- and GAD1-immunopositive interneurons. A systematic study of DNMT1 immunohistochemistry in mouse brain established that this colocalization extends to motor cortex, piriform cortex, striatum, CA1 region of the hippocampus, dentate gyrus, and basolateral amygdala (BLA) interneurons [50]. Analysis of DNMT levels in SZ and BP+ patients compared with control (CON) subjects showed that both DNMT1 and 3A are increased in cortical γ-aminobutyric acid (GABA) neurons of BA10. Unlike DNMT1, DNMT3A is overexpressed only in layers I and II [51]. DNMT3B mRNA was not detected in any cortical layer. The overexpression of DNMT1 and reduced expression of RELN and GAD1 suggests that their relationship might be causal. This was established experimentally using laser capture microdissection of cortical neurons [52]. Results in support of the idea that DNMT1 overexpression mediates RELN promoter hypermethylation in SZ postmortem brain were reported at about the same time [53,54]. Interestingly, both DNMT1 and DNMT3A mRNAs are overexpressed in peripheral blood lymphocytes of SZ patients as well as brain [55].

DNA methylation biomarkers

As noted above, the finding of increased levels of DNMT1 and 3A in peripheral blood cells of SZ subjects begs the question as to whether DNA methylation patterns might be abnormal in blood as well. This interesting possibility is relevant to discussions of epigenetics in psychiatry as blood is readily accessible from patients and is often drawn in the course of treatment. A key issue related to the relevance of biomarkers obtained using blood is the extent to which methylation profiles in blood and brain coincide at specific loci. It was recently demonstrated that many of the key enzymes

involved in DNA methylation and demethylation pathways are present in peripheral blood lymphocytes [55]. However, the finding of methylation/demethylation enzymes in blood is secondary because if specific methylation profiles in blood are shown to be predictive for psychiatric disease, then it should not matter whether these enzymes are also present in brain. In other words, the determination of differential methylation profiles in DNA is independent of any observations made in brain if they can be used to discriminate SZ patients from controls. Genome-wide methylation profiles are cell-type specific, so experimentally determined profiles derived from brain tissue likely represent a composite of the cell types present in the tissue blocks (or sections) used for study. This suggests that any methylation profiles determined using brain tissue will be composites based on the profiles present in multiple neurons and glia. What may be a more important question is whether methylation profiles present in isolated blood lymphocytes from patients reflect the presence of psychiatric disease. This is an old question and at the heart of the matter is whether lymphocytes actually reflect brain function. However, this notion is less important if DNA methylation patterns have diagnostic or predictive value. Moreover, this also opens the door to the question of whether blood DNA methylomes have value in predicting responses to specific medications. Transcriptional differences in interleukin-11 (IL-11) following antidepressant treatment correspond to clinical responses in major depressive disorder (MDD) patients [56]. A recent study shows that DNA methylation in a CpG island in IL-11 might be useful in identifying patients responsive to certain antidepressants [56]. Mathematically, any combinatorial model that exhibits predictive capacity has therapeutic value irrespective of its ability to predict alterations in brain function.

The possibility that methylation of DNA isolated from blood might be useful in predicting drug responses was examined in C57BL/6 mice treated with typical antipsychotics. Methyl-domain-binding 2 enrichment sequencing (MBD-seq; MethylMiner™) was used to examine methylation profiles of mice treated chronically with haloperidol and non-treated mice of the same genetic background [5]. A slow-release pellet was implanted in inbred mice (C57BL/6-treated) to deliver haloperidol at a constant rate and in the range of a therapeutic dose (~5 mg/kg/day) over a 28-day period [57]. Although the better control would have been to implant a control pellet (releasing saline), non-treated mice were not implanted but sacrificed at the same age. Blood and brain (hippocampus, cortex) tissues were isolated and flash frozen on dry ice prior to DNA isolation [57]. The MBD-capture method, which allows for an enrichment of methylated CpG DNA, and MBD-seq, were subsequently performed. This experiment differentiates between what the authors described theoretically as the "signature model" compared with the "mirror-site model." In the "signature model," methylation in blood cells occurs because a disease-related event alters methylation that is detected in blood. In the "mirror-site model," methylation marks in blood would be associated with a phenotype that correlates with corresponding marks in brain. There may be several reasons why "mirror-site" methylation appears in blood, but one plausible mechanism proposes that blood contains cells that are modified as they pass through the brain. In this case, these epigenetic marks mirror those in brain and reflect the etiology of the disease. The purpose of treating animals with haloperidol was not to study the effects of the drug *per se*, but rather to introduce an epigenetic modifier using the typical antipsychotic haloperidol [58]. Interestingly, haloperidol alters the methylation status of ~42% of sites in at least one of the regions in brain (hippocampus and cortex) and also 21% of sites in blood [57]. Comparisons of the genome-wide sequencing data provide insights that reflect aspects of both models. That is, factors, such as haloperidol, that largely impact brain function leave biomarker signatures such as differentially methylated positions (DMPs) in blood. The authors also showed that many DMPs present in blood mirror sites in brain. This suggests that using blood as a

proxy for methylation changes in brain may be a promising approach. The use of inbred mice ensures that animals are genetically identical, which limits the applicability of these data. In general, as investigators begin to look more closely at genome–epigenome interactions, it appears that not only does the epigenome impact the genome but also that the genetic background can have a pronounced effect on the epigenome [59].

Genome-wide methylation studies

The first complete analyses of mammalian DNA methylomes (complete genome-wide sequence including the locations of 5-mC) were published shortly after next-generation sequencing (NGS) became available. Bisulfite is used to chemically modify non-methylated Cs to form Ts while 5-mCs remain Cs [60]. Human embryonic stem cells (ESCs) and fetal fibroblasts were among the first methylomes to be examined at base resolution [21]. This study used MethylC-seq and reported that there were some 61 million 5-mCs in H1 ESCs and 45 million 5-mCs in the fetal fibroblast cell line IMR-90. Some of the first looks at human methylation maps provided considerable anticipation that cellular memories of interactions with external environments (possibly physical, dietary, hormonal, or chemical) might be stored in the methylome [70]. Early reports show high amounts of non-CG methylation present. Gene bodies (i.e., within the gene body) contain higher amounts of non-CG methylation than do promoters and (either 5′ or 3′) flanking regions. Moreover, there was a significant enrichment of non-CG methylation on the antisense strand in gene bodies and this methylation positively correlated with gene expression [21]. It also seemed that non-CG methylation was absent from differentiated cells or could be induced following experimental reprogramming (induced pluripotent stem cells), suggesting a possible role in maintaining pluripotency. The high levels of non-CpG methylation were also observed in a recent analysis of the adult human frontal cortex and at various stages of mouse cortical development [64]. Of the total amount of 5-mC, only ~22% is thought to be dynamic during development, and most of these 5-mCs are located distal to TSSs [71]. Dynamic methylation refers to methylation that changes either spatially or temporally. Many 5-mCs are either always methylated or do not change. Dynamic CpGs colocalize with enhancers and transcription factor binding sites that may be cell or lineage specific. In addition, and as discussed in more detail below, the brain contains the highest amounts of 5-hmC, which can reach as high as 40% of the total methylated DNA fraction in cerebellar Purkinje neurons [72]. As researchers sequence the epigenome with increasing sophistication and attention to modified bases (such as 5-hmC, 5-fc, and 5-caC bases), new information is becoming available that is consistent with a model for tight regulation of DNA methylation and a high degree of integration with other biological processes including gene expression/gene silencing, imprinting, cellular identity, and lineage specification.

Genome-wide array-based methods

Early microarray-based “genome-wide” studies of DNA methylation showed that functionally important DNA methylation occurs not strictly in CpG islands but rather in the CpG island shores [27,73]. CpG island shores are regions that span CpG islands from 500 to 2000 bps on both the 5′ and 3′ sides of the island (see Figure 10.2). Considering the CpG island as a land mass, then on either side of the island are

the so-called shores while further out are the island shelves (2–4 kb from the island). CpG island shore methylation is inversely correlated with gene expression, at least in certain cancer cell lines. The studies mentioned above focused on a large set of CpG sites designed for detecting changes in tissue-specific DNA methylation across the genome. Array-based approaches for detecting methylation allow for the screening of large numbers of CpGs inside and outside of CpG islands across the genome. Methylation arrays do not assay all CpGs in the genome and have additional weaknesses (see below), but they provide a wide and unbiased coverage of CpGs (~4.6 million) that have been implicated in gene regulation and tissue-specific differential methylation. By assessing the methylated sites in CpG islands and shores (at variable distances), it has been shown that both hypo- and hypermethylation of DNA in cancers are located at varying distances along the CpG island shores. Moreover, in the tissues examined (brain, liver and spleen), most tissue-specific differential methylation also occurs in these regions [27]. By performing NGS of DNA enriched in methylated DNA (MeDIP-seq) from brain, liver, spleen, and lung, another study showed that tissue-specific CpG island methylation is prevalent in gene bodies and not in promoter regions [46]. These findings are interesting and somewhat novel but represent a departure from the notion that promoter methylation is a primary mediator of gene regulation. It has been suggested that correlations between genome-wide methylation patterns and gene expression data are not strong because currently existing bioanalytical methods oversimplify and do not capture the complexity of existing methylation patterns [74]. High-resolution DNA methylation profiles (methylation fingerprints) that correlate with gene expression changes have been generated from genome-wide sequencing data by examining entire differential methylation profiles in the vicinity of promoters [74]. In other words, correlations between promoter methylation patterns and mRNA expression become evident when the promoters are first classified based on methylation signatures. This allows for a refined approach that more readily finds correlations as there appear to be many types of methylation signatures, not all of which correlate with expression data.

As noted above, genome-wide studies of DNA methylation have been carried out using blood and postmortem brain tissue of psychiatric patients. With the advent of high-density methylation arrays (BeadChip™ arrays; NimbleGen), novel restriction enzyme-enriched assays, affinity enrichment-based techniques (such as MeDIP and hMeDIP), and NGS analysis of bisulfite-treated DNA samples (Bis-seq or MethylC-seq), the amount of information that requires processing is quite large [75]. For the array datasets, normalization is critical for comparison of results between samples. Moreover, commercially available methylation arrays that measure levels of hydroxymethylation are only recently available. The Illumina® Infinium® HumanMethylation450K BeadChip™ DNA methylation arrays provide wide coverage of CpGs (~96% of UCSC-listed CpGs) and cover 99% of RefSeq genes (National Cancer Biotechnology Reference Database), and the CpG probes are distributed across whole gene regions including promoters, gene bodies, and 3′ untranslated regions. Each probe on the array coincides with a single CpG and the arrays have been tested extensively [63,76,77]. The drawbacks of using methylation arrays are that: (1) they do not allow for the measurement of hydroxymethylated CpGs; (2) they do not measure CpH methylation, which has been shown to represent as much as 53% of the total methylation fraction in neurons [64]; and (3) the CpG probes on the array are selected by a team of specialists based on existing knowledge of DNA methylation. Although this latter point may not seem negative, any platform that does not have complete CpG coverage is biased based on subjective opinions. The choice of array probes has been based on previous studies showing relevance to gene regulation. Algorithms are also now available for preprocessing of the HumanMethylation450K array data to minimize variance and improve statistical power, which can be

particularly useful for the detection of small changes in methylation likely associated with complex disease phenotypes [78].

Algorithms have been developed to map and align peaks for NGS datasets. In addition, for those analyses that utilize brain tissue, strategies such as bump-hunting, can be used to detect differentially methylated regions and to eliminate experimental variability [79]. However, there are no generally accepted standards for clinical studies, and difficulties are often encountered when comparing results from methylation arrays (Illumina Infinium Methylation BeadChips), whole genome or reduced representation Bis-seq, and the more comprehensive MethylC-seq. In addition, many of the afore-mentioned approaches do not accommodate copy number variants that have been observed in many genetic studies. As the use of DNA methylation profiling moves toward diagnosis, prognosis, and drug response prediction, approaches need to be centralized and better biomarkers need to be developed and replicated. It is also necessary to have large numbers of reference datasets available for comparisons [75]. Because the DNA methylation/hydroxymethylation landscape is representative of how the environment impacts the genome, it might be expected that individual phenotypes associated with psychiatric illness are differentially represented in the epigenome. It seems entirely plausible that the heterogeneity observed during clinical presentation (e.g., SZ or autism) and during disease progression might be reflected in DNA methylation/hydroxymethylation profiles. The key to understanding this experimentally involves knowing the best method to use to look for differences and in what tissue or cell type to look (see also Box 10.1).

Methylation of BDNF in psychiatric disorders

Brain-derived neurotrophic factor (BDNF) is a secreted protein and a member of the neurotrophin family of growth factors that provide trophic support for neurons and encourage the growth and differentiation of new neurons and synapses. The importance of DNA methylation in regulating the rat BDNF gene was shown by the correlation between expression in response to activity-dependent chromatin remodeling and decreased CpG methylation within the regulatory region of the gene [83]. It was also shown to be a calcium-dependent target of the methyl-domain-binding protein MeCP2 [84]. The DNA methylation patterns corresponding to *BDNF* have been studied for some time in mouse, rat, and human. Human *BDNF* is organized as a complex series of as many as 10 non-coding promoter exons that are spliced to the same primary downstream coding exon [85,86]. Differential promoter use during development and in different brain regions has been mapped in postmortem brain tissue from individuals of different ages [86]. Moreover, the differential sensitivity of the mouse and rat promoters to epigenetic regulation has been described [87]. The sensitivities of the human BDNF promoters to antidepressant medications indicate that the four most abundant promoter/exon transcripts (I–IX, II–IX, IV–IX, and VI–IX) (Figure 10.5) are significantly upregulated in SZ patients treated with antidepressants [88]. However, this study also reported that there is little relationship between antipsychotic drug use and BDNF mRNA levels. Of note, as translation initiation starts in exon IX, each of the above promoter/exon combinations specifies the same BDNF protein.

Human BDNF mRNAs and protein are consistently downregulated in postmortem tissue isolated from patients with psychiatric diseases. It has been suspected for some time that BDNF expression correlates with specific promoter methylation patterns in brains of patients with psychosis [89]. Moreover, a number of studies have shown reduced BDNF levels in blood [90,91]. In search of a potential methylomic biomarker, genomic DNA from peripheral blood of Japanese patients diagnosed with MDD was

BOX 10.1 NEXT-GENERATION SEQUENCING

In terms of methodologies, quantitative differences observed by different investigators are often dependent on the means of detection. For example, enrichment techniques, such as methylated DNA immunoprecipitation (MeDIP) and hydroxymethylated MeDIP (hMeDIP), often show larger differences than what is commonly observed in genome-wide methylation studies. This can be, in part, a function of the amplification steps used in the two technologies. Both approaches afford appropriate quantitation at specific genomic locations and are internally comparable (relative quantitation). The polymerase chain reaction (PCR) used in NGS assays employs a linear amplification method that amplifies all fragments in the genome with approximately the same efficiency. In contrast, when the levels of a single gene are examined (using MeDIP, hMeDIP, or ChIP), the PCR amplification steps are exponential and should be normalized and compared with the levels observed of the same amplicon in different conditions (e.g., SZ compared with CON).

The haploid human genome contains an estimated 29,000,000 CpGs, each of which represents a putative site for DNA methylation. Taking into account that non-CpG methylation represents a considerable amount of the total fraction of methylated DNA, the potential number of sites is considerably higher. A large number of techniques have been developed over the years to examine regional and genome-wide variations in DNA methylation [80,81]. For a summary of current methods and terms, including those used for measuring 5-hmC, see the Abbreviations list and the Glossary. Massively parallel sequencing of DNA with commercially available NGS platforms is generally conducted in several steps. The chemistry and specific features of each method vary somewhat depending on the sequencer manufacturer. Shotgun DNA sequencing libraries are generated from fragmented DNA and, following adaptor ligation, DNA PCR is amplified *in vitro*. The DNA is then sequenced by synthesis, such that the order of bases is determined by the addition of nucleotides to the complementary strand. The spatially segregated, amplified DNA templates are sequenced simultaneously in a massively parallel fashion without the requirement for a physical separation step. The chemistry allows for simultaneous sequencing of multiple "bar-coded" DNA templates. Running multiple samples at the same time coupled with the use of emulsion-based PCR, bridge amplification, and single-molecule sequencing, as well as the use of different chemistries, all have increased sequencing speeds and tremendously reduced costs. One of the key conceptual breakthroughs was the coupling of sequencing machines with high-speed computers to allow for direct data uploading and subsequent data alignment. By randomly generating short DNA molecules containing overlapping stretches of DNA sequence that are sequenced multiple times, error rates at specific positions have gone down considerably. The Human Genome Project (HGP) was an international scientific research project that was budgeted by the U.S. government beginning in 1987. The announcement of completion of the draft sequences by the HGP and Celera Genomics occurred in 2000, followed by publication of the draft human genome in 2001. By comparison, the publication of the first complete human genome at 7.4× sequencing coverage (redundancy) using NGS technology took approximately months [82]. Making this comparison vastly underestimates the huge amount of effort involved in reaching this milestone. However, it also highlights the advantages of approaching the problem using comparatively short sequence reads coupled with advanced computer technologies for assembly.

used to analyze the methylation of BDNF promoters I and IV [92]. This study used the Sequenom® MassARRAY® high-throughput analyzer to assess the methylation profiles of a stretch of 1092 bp (containing some 81 CpGs: promoter 1) and a 1137-bp stretch containing 28 CpGs using bisulfite-converted patient DNA. The study showed that the methylation profiles of BDNF promoter I matched the patient classification (healthy controls, $N = 18$; MDD patients, $N = 20$) based on clinical diagnoses [92]. Another study evaluated the efficacy of BDNF exon IV in leukocytes of MDD ($N = 20$ female, 19 male) patients to predict antidepressant treatment response [93]. Of the sites analyzed (13 total), the baseline methylation status of position −87 (BDNF promoter IV) predicted antidepressant non-response [93]. The authors hypothesized that antidepressants reduce the binding of MeCP2 to BDNF promoter IV by increasing the levels of MeCP2 phosphorylation. The size of the experimental groups in these latter studies were small and have yet to be replicated. Contributions of environmental factors, including age,

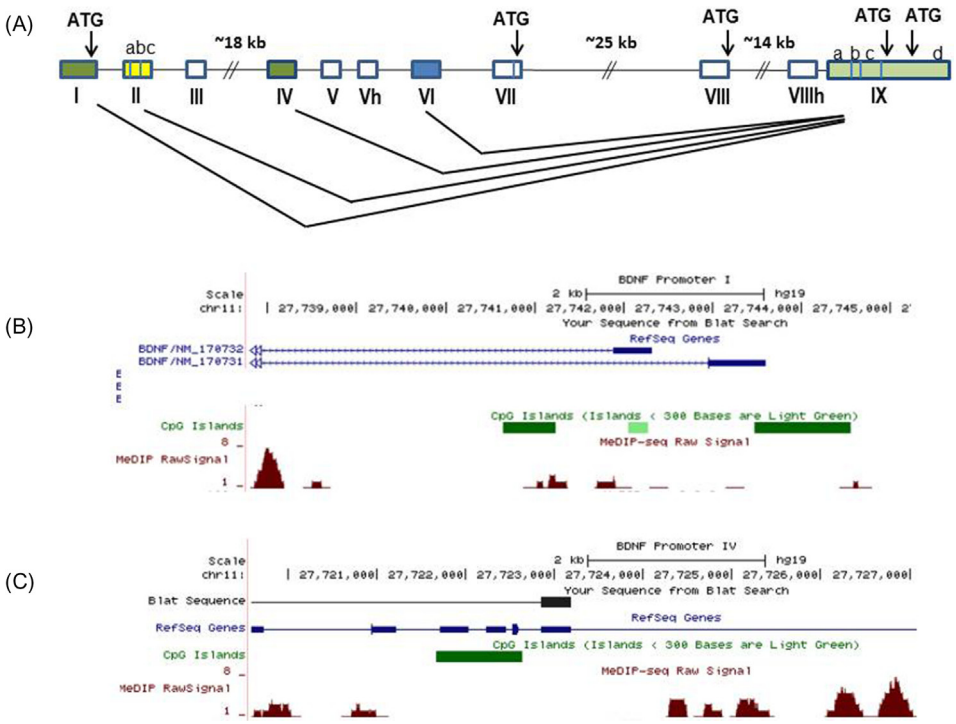

FIGURE 10.5

Structure of *BDNF*. (A) The gene for BDNF is illustrated (from the UCSC genome browser) based on exon numbering [86,88]. Exons I, II, IV, and VI represent the major contributors to BDNF mRNAs in the adult brain. These are spliced to the same coding sequence in exon IX, making identical proteins (see joining lines). There are additional translational start sites as well. Large intronic gaps are shown in numbers along the *BDNF* gene. Exons II and IX are further subdivided (see letters). (B) Coordinates (in UCSC Browser format versus HG19) of BDNF promoter (exon) I along with corresponding mRNAs (transcription is on antisense strand moving left) with the various RefSeq (NCBI, NLM, NIH database) numbered transcripts (NM_170732 and NM_170731). CpG islands are shown in boxes below the transcripts and the MeDIP-seq raw methylation based on data from the human brain [46]. The amount of methylation in each region is proportional to the height of the signal. (C) The same information for BDNF promoter IV. The scales for panels B and C are the same (as shown). *BDNF* promoters I and IV are most commonly examined with respect to changes in DNA methylation. *BDNF* promoter IX has been used in studies as well. The indicated Blat sequence represents the input sequence used to find the corresponding genomic regions [153]. For promoter I the 5′ most portion of NM_170731 was used, while for promoter IV, the 5′ most portion of NM_170733 was used to Blat UCSC HG19. The position of the sequence in (B) indicates the 5′ portion of promoter IV, which extends further to the right. These panels illustrate the locations of regional methylation in the vicinity of these promoters in normal human brain.

illness duration, diet, medication history, and exposure to stress *in utero*, in addition to genetic background and race, may play an important role in determining the utility of blood BDNF methylation levels as biomarkers for MDD [94]. However, it seems likely that factors relevant to psychiatric diseases such as MDD appear reflected in the methylation profile at the BDNF locus, which may therefore provide useful information in the context of prognosis.

In schizophrenia, BDNF mRNA expression is reduced in multiple cortical areas and in the hippocampus [86,88,95]. DNA isolated from peripheral blood cells of healthy subjects ($N = 100$) and SZ patients ($N = 100$) was recently used to assess the DNA methylation patterns corresponding to specific CpGs in BDNF I and IV promoters using pyrosequencing [96]. Pyrosequencing is used to differentiate between 5-cytosine (5-C) and 5-mC over short stretches of DNA. The data showed that the methylation of specific promoter I compared with IV CpGs may be associated with the pathophysiology of SZ [94]. Another recent study of parietal cortex showed higher levels of 5-mC and 5-hmC in BDNF promoter IX in DNA isolated from psychotic patients compared with controls [97]. Other studies showed that patients with psychiatric disorders have decreased amounts of neuronal BDNF, which correlates with increased levels of methylation at selective CpGs in BDNF promoter IV and not promoter I [96]. In many cases, the methylation differences at specific sites between psychiatric groups is not large but is selective for specific CpG sites [6]. Moreover, the differences are consistent across both brain and peripheral blood [94]. Similar observations regarding the methylation of BDNF have been described with respect to brain and plasma of suicide victims [98].

Array-based studies for SZ

One of the first comprehensive methylation array studies was carried out on DNA isolated from human postmortem cortex of the Stanley Medical Research Institute brain collection [89]. The DNA used in this study was first enriched for the non-methylated fraction using methylation-sensitive restriction enzymes such as *Hpa*II and *Mcr*BC. The use of the non-methylated fraction of DNA was shown in a previous study to improve the chances of detecting methylation differences [99]. Samples were hybridized to custom-made CpG island arrays using DNA from SZ ($N = 35$), BP ($N = 32$), and CON ($N = 28$) subjects [89]. The analysis was done so as to maintain gender-specific signals and signals that overlapped in both males and females. Functional network analysis of the results showed significant changes in a number of genes associated with both glutamatergic and GABAergic neurotransmitter pathways [89]. Moreover, there were a number of genes that had been previously linked to major psychosis, including *GRIN3B* and *GRIA2*. This study also provided moderate evidence for an association between BDNF genotype at a non-synonymous single nucleotide polymorphism (SNP) and methylation, suggesting that DNA sequences can influence epigenetic marks. The authors reported no evidence for association between catechol-*O*-methyltransferase (COMT) promoter methylation and psychosis (PSY). COMT has been genetically linked to SZ and PSY [100]. These studies prompted an additional set of experiments examining both blood and brain tissue from psychiatric subjects [101].

A recent methylation array study (Illumina® Infinium® HumanMethylation27 BeadChip™) that was performed using blood from 98 SZ patients and 108 healthy CON subjects identified a small number of differentially methylated CpGs [102]. Eleven of the CpG sites significantly correlated with

symptoms associated with reality distortion. Pathway analysis of the corresponding genes showed that they were associated with the inflammatory response [102]. Another recent study examined peripheral blood cells from first-episode SZ patients ($N = 17$; 15 normal controls) also using the Illumina Infinium HumanMethylation27 BeadChip array [103]. One advantage of first episode cohort is that confounding variables such as disease duration (first psychotic episode and subsequently diagnosed with SZ within 60 months) and antipsychotic medication history (less than 16 cumulative weeks of antipsychotic use) are somewhat controlled. Results from the analysis provide evidence that differentially methylated CpGs are particularly abundant in CpG islands and are enriched in genes associated with the nuclear lumen, transcription factors, and nucleotide binding factors. Analysis of the array data showed that 603 CpG sites were significantly different, many of which were hypomethylated in first-episode patients. Of the significant differentially methylated regions, 96.4% were located in CpG islands [103]. Compared with similar previous studies, this report identified *HTR1E* and *COMTD1* as functionally related to SZ. Related genes, such as *HTR1A* [104], *HTR2A* [105], and *COMT* [58,106], were not identified but previously shown by others to be differentially methylated in SZ. One problem in comparing different studies is that sample sizes need to be (nearly) equivalent. In the study just described, the sample size was relatively small [103]. Racial factors (all subjects were Japanese) also impact the epigenome; this factor is currently understudied, and although the patients were newly diagnosed the exclusion criteria stated that patients be diagnosed within 60 months of having their first episodic break [103]. True first-episode psychosis patients are likely more difficult to enroll in studies like these particularly in the absence of a SZ diagnosis.

In addition to the analysis of affected patients and controls, twin studies have advantages for examining epigenetic marks. Evaluating DNA methylation (and other epigenetic marks) in twins discordant for psychiatric disease (or other factors) allows for the control of genetic background. Studies on how genetic background impacts epigenetic marks or whether the same marks are written precisely the same way in a different genetic background are only just beginning. Comparing DNA methylation patterns in twins discordant for psychosis or SZ allows for the elimination of what might have a profound impact on either the writing of these marks or how they are read. A recent analysis was performed on peripheral blood samples from 22 twin pairs discordant for SZ using the Illumina Infinium HumanMethylation27 BeadChip array [107]. A large number of regions showed SZ-associated DNA methylation differences between twins, with the top differentially methylated region (DMR) being in the promoter of *ST6GALNAC1*. Among the top 100 SZ-associated genes identified in blood from twins discordant for psychosis and the 589 top genes identified in first-episode (or newly identified) SZ subjects, there was only one common gene, *ADAMTS3* (ADAM metallopeptidase with thrombospondin type 1 motif, 3). The data show hypomethylation of probes at this locus. The functional significance of this gene in the context of SZ, PSY, or even neuronal function is currently not known but there has been at least one study to show that a *de novo* mutation in the gene is associated with a case of sporadic SZ [108]. The methylation changes observed in twins may not be expected to be in common with the methylation marks in independent SZ subjects. The heterogeneity observed in clinical presentation, disease time course, and symptom progression make methylation comparisons between sets of twins or between different clinical cohorts complex. More importantly, as the hunt for biomarkers in blood or lymphocytes of psychiatric patients begins to include larger numbers of subjects (as seen with genetic studies), researchers will be wise to better match confounding variables in cohorts with respect to illness duration, medication profiles, race, and gender before analyzing the data.

Second generation methylation arrays and SZ

More recently, a study was reported on the analysis of DNA from the blood of SZ patients that analyzed over 480,000 CpG sites using the second-generation Illumina Infinium HumanMethylation450 BeadChip array [109]. This is perhaps the most comprehensive array kit currently available and is currently the industry standard for DNA methylation arrays. Although arrays provide coverage for over 485,000 CpGs, the total number of CpGs in the genome is considerably higher (~29,000,000 total CpGs). This study [109] identified a large number of DMRs in two cohorts of Japanese SZ subjects. It was performed on peripheral leukocytes from a cohort of medication-free SZ patients (24 medication-free SZ for at least months) and controls (23 CON), and three pairs of monozygotic twins discordant for SZ. Combining the data from both sets of studies provided a total of 234 CpG sites common among the two cohorts, and a significant fraction (40.6%) were in CpG islands with a smaller amount (13.3%) in CpG island shores. The most significant gene region associated with CpG methylation in SZ subjects occurred in CpG-island-containing promoters. This finding contrasts with earlier reports discussed above showing that DNA methylation important to gene regulation occurs in CpG island shores [22]. Overall, comparisons of results from these studies indicate that there is little overlap between top CpGs or the genes identified [101–103,109].

The use of different methylation array platforms (Illumina Infinium HumanMethylation450k versus HumanMethylation27k), sample preparation protocols (leukocytes compared with whole blood), phenotypes (medication-free patients compared to with medicated subjects), or different analytical (normalization/statistical) methods likely explains the lack of concordance between findings from the different studies. Of the above factors, perhaps medication history (type and duration) contributes to the largest amount of variability. As previously reported [110], the DNA methylation profiles derived from whole blood represent a mixture of cell types including several leukocyte subtypes. The studies noted above used different versions of the methylation arrays, and, after correcting for quality controls, only 7562 probes were evaluated [102]. Finally, although the study controlled for current medications (in chlorpromazine equivalent doses), chronic medication history was not available. It has been shown that various antipsychotics taken chronically have an effect on DNA methylation [111,112]. A large number of studies that have examined DNA methylation in various brain regions and in peripheral cells have been recently reviewed [113].

Enrichment sequencing for studies of SZ

Numerous methods have been developed to enrich specific sequences prior to large-scale sequencing. For example, MBD-seq (e.g., MethylMiner; Invitrogen, CA) and MeDIP-seq are two approaches that have been used to capture methylated DNA to enrich for a subset of sequences in the genome that are differentially methylated. These two approaches were recently compared, and both approaches were shown to be sensitive to the detection of differentially methylated regions [114]. Whereas MeDIP-seq enriches methylated regions with low CpG density, MBD-seq tends to be more efficient for regions of higher CpG density including CpG islands. MeDIP-seq detects methylated Cs whether or not they are in CpGs, but MBD-seq does not. This is because MeDIP relies on 5-mC immune precipitation of fragmented DNA, and MBD enrichment uses the methyl DNA-binding domain of MBD2, which has a high affinity for 5-mCpG sequences. An efficient and robust analytical pipeline (for MBD-seq) was recently

proposed and evaluated for use in large-scale studies [115]. This was recently used to evaluate the methylation profiles of 1500 SZ case and control blood samples. While the entire dataset was only recently published [116], at least two differentially methylated regions (corresponding to *FNDC3B* and *DCTN*) replicated with highly significant *p* values in independent sample DNAs from the same study populations. Using genome-wide association studies of age-related methylation changes, the authors confirmed a previously reported association with *GRIA2* [115].

The use of large numbers of samples coupled with the development of new software approaches to analyze genome-wide data may allow for more precise determinations of potential diagnostic methylation biomarkers in peripheral blood cells. This also raises the interesting possibility that blood taken and stored for long periods of time might be assayed for genome-wide methylation levels several years after the samples are taken. For example, neonatal blood samples taken worldwide (on Guthrie cards) from newborns of psychiatric patients may prove to be a new source of DNA for genome-wide methylome studies [117]. Neonatal dried blood samples are routinely used for genotyping studies with excellent results. The proof of concept for this type of approach was recently carried out by profiling bisulfite-treated DNA isolated from 3.5-year-old dried blood spots on the Illumina® Infinium® HumanMethylation27 BeadChip™ arrays [117]. Both adult and neonatal blood samples were spotted and stored, and 26- to 28-year-old blood cards from the Danish Newborn Screening Biobank were used in the study. After assessing quality control probes on each array (e.g., bisulfite conversion controls, hybridization controls, negative controls, efficiency controls), the samples showed some ~50 CpGs significantly different between adult and neonatal samples. Moreover, comparisons of the data showed that despite 26–28 years of storage, samples extracted from stored Biobank cards served as a suitable source for profiling. As the field of epigenetic psychiatry moves toward standardizing the procedures used for methylation profiling of clinical samples, whether fresh or archived, it seems clear that appropriate methods need to be established for: (1) collecting samples, (2) extracting DNA, (3) the genome-wide methylation analysis platform (whether Bis-seq based, MethylC based, or methylation array based), and (4) data analysis/bioinformatics and quality control measures. Moreover, we must make decisions on whether to use isolated DNA from whole blood, leukocytes, or thrombocytes (platelets). An additional possibility is to isolate the separate components of leukocytes such as lymphocytes, monocytes, different granulocytes, etc. As one example, the DNA methylome of human peripheral blood mononuclear cells was recently reported which revealed a rich epigenomic landscape for over 20 distinct genomic features [118]. These decisions will involve rigorous testing at multiple laboratories prior to implementation and notification of the scientific community, similar to what was recently done reported by the ENCODE Consortia Data Release, Data Use, and Publication Policies.

Genome-wide studies of methylation in ASD

Prior to looking at ASD-specific changes in methylation, the methylation profiles corresponding to DNA isolated from nine control individuals with no evidence of neuropathological disease or neuropsychiatric disease were determined [119]. This study compared the MeDIP-seq methylome profiles in various brain regions (inferior frontal gyrus, middle frontal gyrus, entorhinal cortex, visual cortex, temporal cortex, and cerebellum). Bisulfite pyrosequencing was used to confirm DNA methylation estimates at various areas across selected regions in additional brain and blood samples. The data show that CpG

islands are significantly hypomethylated compared with CpG island shores and gene bodies [119]. In addition, while a large number of CpG island promoters are hypomethylated, promoter methylation is inversely correlated with CG density, with low-density promoter regions being the most methylated of any feature tested [119]. Tissue-specific differentially methylated regions (TS-DMRs) are significantly enriched near genes involved in functional pathways related to neurodevelopment and neuronal differentiation, including *BDNF*, *BMP4*, *CACNA1A*, *CACA1AF*, *EOMES*, *NGFR*, *NUMBL*, *PCDH9*, *SLIT1*, *SLITRK1*, and *SHANK3*, among others. Collectively, this study used a combination of MeDIP-seq along with Illumina Infinium HumanMethylation450 BeadChips to demonstrate that between-tissue variation in DNA methylation greatly exceeds between-individual differences within any one tissue. This indicates that peripheral tissues may have utility in providing informative biomarkers for complex neurobiological phenotypes [119].

Autism spectrum disorder is a common neurodevelopmental disorder characterized by impairments in social interaction, communication, and restricted or repetitive behavioral patterns. ASD represents a collection of neurodevelopmental phenotypes that share features with other disorders such as Rett syndrome, Angelman's syndrome, fragile X, and MeCP2 duplication syndrome. Rett syndrome, which is due to mutations in *MeCP2*, is pertinent to the current discussion because MeCP2 is an abundant methyl-DNA-binding protein. While heritability estimates for ASD are high, conventional genetic efforts have yielded a number of reproducible candidate genes that account for only a low percentage of affected individuals. Environmental factors that involve epigenetic mechanisms likely play an important role in the etiology of this psychiatric disease. A large number of papers document the search for factors that regulate individual genes implicated in ASD. DNA methylation at multiple loci including the oxytocin receptor [120] and SHANK3 [121], among others [122,123], have been implicated in the regulation of ASD risk genes. Another recent publication shows that MeCP2 binds CpGs containing 5-hmC with comparable affinity to 5-mCpGs [124]. This suggests the possibility that Rett syndrome might be associated with an imbalance in the amounts of 5-mC and/or 5-hmC in specific gene regulatory regions. Although ASD does not typically involve a mutation in MeCP2, there is evidence that the levels of MeCP2 or MeCP2 binding at specific CpGs may be altered in ASD [125].

Two recent studies were carried out that examined whether genome-wide methylation fingerprints might exist in distinct brain regions of ASD patients. The first of these studies used the Illumina Infinium HumanMethylation27 BeadChip to examine common sites of methylation in DNA isolated from the blood of multiple monozygotic twins discordant for ASD and related phenotypic traits [126]. Participants were recruited for the study from the Twins' Early Development Study based on responses by parents and teachers to Childhood Autism Symptom Test measures. As noted previously, by comparing twin pairs discordant for ASD, results due to differences in genetic background are minimized while the contributions of environmentally driven epigenetic events should play a more dominant role. A genome-wide analysis of monozygotic twin pairs discordant for ASD ($N = 50$, 100 individuals) showed that specific methylation patterns associated with autistic symptom severity are observed at multiple loci previously implicated in the autistic phenotype such as *AFF2*, *AUTS2*, *GABRB3*, *NLGN3*, *NRXN1*, *SLCA4*, and the imprinted locus *UBE3A* [126]. Quantitative autistic trait scores correlate with DNA methylation at multiple CpG sites [126]. A second recent study examined DNA methylation among three brain regions including the dorsolateral prefrontal cortex, temporal cortex, and cerebellum of 19 autism cases and 21 unrelated controls [127]. The group used the

Illumina Infinium HumanMethylation450 BeadChip arrray to look for differential methylation across brain regions. The authors used "bump hunting," which assigns probes to clusters such that adjacent or neighboring probes are separated by no more than 500 bp [128]. This effectively narrows the areas of the genome that are searched, providing differentially methylated regions (DMRs) instead of differentially methylated positions (DMPs). Four statistically significant DMRs were identified in the study, and three of these four were replicated in a different set of samples and across different brain regions [127]. The magnitude of the methylation changes between groups is on the order of 6.6–15.8%, which is comparable with the differences often reported in psychiatric disease [89,129]. These regions include: (1) a DMR located near the 3′ UTR of *PRRT1*, a proline-rich transmembrane protein, that is hypomethylated; (2) a second DMR that resides in the temporal cortex in the promoters of Tetraspanin 32 (*TSPAN32*) and chromosome 11 open reading frame 21 (*C11orf21*); (3) a third temporal cortex DMR located in an intergenic region with the closest gene (*ZFP57*) some 3.5 kb away; and (4) a fourth DMR in cerebellum that is 15.8% more methylated than controls and resides in the succinate dehydrogenase complex, subunit A, flavoprotein pseudogene (*SDHAP3*). By making certain assumptions in carrying out the data analysis, as was done here, and in the absence of corresponding RNA expression studies, it is somewhat difficult to interpret what these data represent in a broader context. Clearly, these studies will benefit from additional replication.

Hydroxymethylcytosine historical perspective

As early as 1953, the genomes of bacteriophages of T2, T4, and T6 were first shown to contain 5-hmC in place of 5-C [130,131]. More recently, the presence of 5-hmC was shown to be present in mammalian brain and is particularly abundant in Purkinje cells of the cerebellum [72]. Using a combination of thin-layer chromatography, high-pressure liquid chromatography, and mass spectrometry, it was shown that 5-hmC represents 0.6% of total nucleotides in Purkinje cells and 0.2% in granule cells, and is abundant in brain. In Purkinje cells, this represents as much as 40% of the methylated cytosine. In an article that appeared in the same journal [132], the enzyme TET1 was shown to catalyze the conversion of 5-mC to 5-hmC. Subsequent quantitative mapping studies showed a differential distribution of 5-hmC throughout the brain [133]. In recent years there has been considerable interest in the observation that hydroxylation of 5-mC is probably the first step in the mechanism by which methylated cytosine is replaced in brain (see Figure 10.1). Subsequent studies have shown that in addition to 5-hmC, TET proteins also convert 5-mC to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) [134] and that base excision is likely mediated by the action of thymine DNA glycosylase [135,136]. New techniques were developed for the detection of 5-hmC and other intermediates (see Figure 10.6). For example, TET1-assisted bisulfite sequencing (TAB-seq) uses the properties of recombinant mouse TET1 to define the locations of 5-hmC with base resolution [137]. Oxidative bisulfite sequencing utilizes the chemical properties of potassium perruthinate ($KRuO_4$) to differentiate 5-hmC from 5-mC following bisulfite conversion [66].

A series of genome-wide mapping studies of ESCs and other tissues followed these discoveries in which the distribution and possible function of 5-hmC were examined [13]. In the context of many of these, function was governed by gene location. For example, the finding that 5-hmC is enriched in enhancers in human ESCs implies a specific role for 5-hmC in gene regulation [138]. It was soon

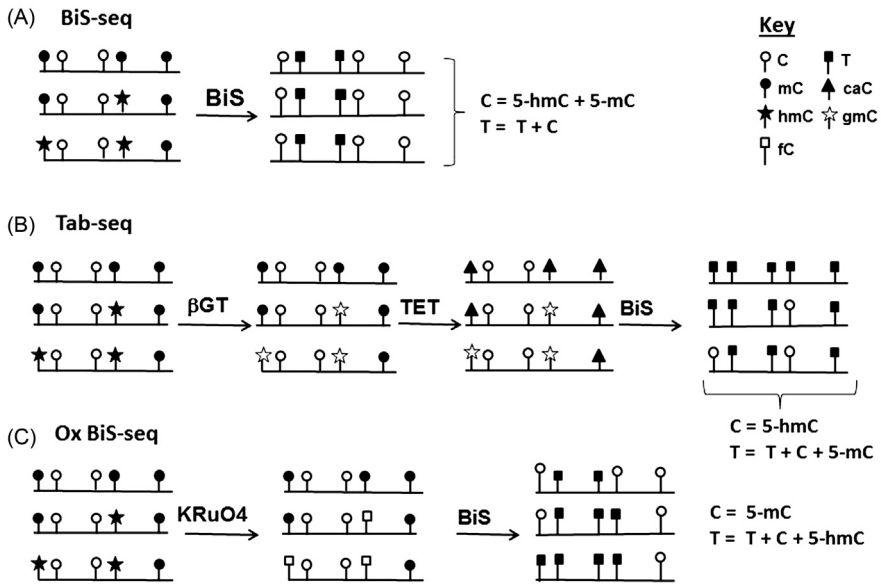

FIGURE 10.6

The effects of (A) bisulfite conversion; (B) β-glucosylation, TET1, and bisulfite treatment; and (C) $KRuO_4$ and bisulfite on the indicated sequence by these methods. Bisulfite converts both Cs and Ts to Ts while 5-mCs and 5-hmCs remain as Cs. With Tab-seq, βGT protects 5-hmCs, converting them to glucosyl-5mCs which after bisulfite treatment remain as Cs. TET1 then oxidizes 5-mCs to carboxyCs which are converted to Ts by bisulfite. Oxidative bisulfite sequencing uses $KRuO_4$ to convert 5-hmCs to 5-fCs, and, following bisulfite, 5-fCs, Cs, and Ts are converted to Ts. These methods are then compared with the patterns obtained with bisulfite treatment alone to determine which base is which at any given position.

recognized that TET proteins play a dual role in repressing transcriptional regulation and in catalyzing the hydroxylation of 5-mC to 5-hmC [139–141]. While we continue to gain new insight into genome-wide distributions of 5-hmC in ESCs and in adult brain structures, the rules regarding binding and occupancy of TETs at specific 5-hmCs remain unclear as does the role of this modification in modulating transcription. In addition, 5-hmCs may be targeted by the growth arrest and DNA damage-inducible protein GADD45 for removal by the interaction of members of the AID/APOBEC family of 5-hmC deaminases and the base excision repair (BER) pathway including thymine deglycosylase. Moreover, 5-fC and 5-caC are stable and committed intermediates derived from 5-mC, although their abundance is markedly lower in all tissues including brain [142,143]. We now know that CpGs in so-called CpG islands and shores can be methylated, hydroxymethylated, and ultimately demethylated depending on the status of various chromatin-modifying proteins proximal to gene regulatory domains. Moreover, as mentioned above, it has been demonstrated that non-CpG methylation (e.g., CpH) is abundant in neurons and plays an important role in gene regulation, as highlighted in a recent analysis of the human and mouse frontal cortex during different development stages [64].

5-hmC in psychiatric disorders

The discovery of high levels of 5-hmC in neurons of the cerebellum, including Purkinje cells, has attracted considerable attention by investigators studying autism. Genome-wide changes in 5-hmC are associated with neurodevelopmental genes in the developing human cerebellum. A recent study of 5-hmC in human cerebellar development showed that differentially hydroxymethylated regions were prominent in genes regulated by the fragile X mental retardation protein, including several autism candidate genes [15]. Another genome-wide study performed on isolated neurons of the mouse cerebellum showed that 5-hmC is depleted near transcriptional start sites but is prevalent in gene bodies, suggesting an inverse correlation between transcription and gene body 5-mC [124]. It has been proposed that there are two populations of MeCP2 present in the brain: one enriched in nucleosomes and the other present in highly accessible, DNaseI-hypersensitive chromatin domains. This suggests that MeCP2 function is pleiotropic and is involved in transcriptional silencing as well as in transcriptional activation and chromatin remodeling [144]. Another study employed quantitative mass-spectrometry-based proteomics to demonstrate the existence of some 260 proteins that bind 5-mC, 5-hmC, 5-fC, or 5-caC in distinct cell types at different developmental times [145]. Some of these proteins are active in binding only ESCs while others are present in adult brain. This suggests that not only are these bases present as DNA demethylation intermediates but that they also each play important and independent roles in cellular function. It also seems clear that the proteins interacting with these intermediates are only beginning to be recognized.

The levels of TET1 mRNA and protein are higher in the inferior parietal cortex and cerebellum of PSY patients, while the levels of activation-induced deaminase/apolipoprotein B mRNA-editing enzymes (AID/APOBEC) remain unchanged [146]. In addition, the levels of 5-hmC are also higher in patients as might be expected if the formation of 5-hmC is proportional to the levels of TET1 [146]. Moreover, higher 5hmC levels were detected at the GAD1 promoter in the PSY group which were inversely proportional to the levels of GAD1 mRNA [146]. These data suggest that either a protein downstream of TET1 is affected in these patients or 5-hmC is a repressive epigenetic mark. We recently performed methylated DNA immunoprecipitation (MeDIP) and hydroxymethylated DNA immunoprecipitation (hMeDIP) and showed that 5-mC and 5-hmC are increased in the promoters of *RELN* and *GAD1* in ASD cerebellum [125]. The enrichment of 5-hmC in the GAD1 promoters was confirmed by TAB-seq [125]. These findings are consistent with the hypothesis that an increase of 5-hmC (relative to 5-mC) at specific gene domains enhances the binding of MeCP2 to 5-hmC, thus reducing expression of the corresponding genes in ASD cerebella. Although these results are still preliminary, investigations of the levels of 5-hmC and 5-mC at specific genes are likely to provide additional information to increase our understanding of molecular processes that underlie the etiology of symptoms underlying psychiatric disorders.

Summary and conclusions

As recent as 2013, new findings that call into question current thinking regarding DNA methylation, methylation writers (DNMTs), and methylation readers (MeCP2) were published. The importance of non-CpG methylation was underscored by a study showing that greater than 50% of the total 5-mC in neurons exists as 5-mCpH and not as 5-mCpG. Whole genome methylcytosine and hydroxymethylcytosine maps at base

resolution show profound changes during frontal cortical development, indicating distinct functions for DNA modifications in a cell-specific capacity. DNMT1 is pleiotropic and known to participate in a wide variety of cellular functions. It was recently shown that DNMT1 interacts with non-coding RNA (ncRNA), such as CCAAT/enhancer-binding protein alpha (CEBPA) ncRNA. This DNMT1–CEBPA/ncRNA interaction inversely links transcriptional regulation to DNA methylation [147]. Expression profiling of transcripts associated with DNMT1 coupled with genome-wide methylation demonstrated the generality of this finding to numerous genes [147]. These findings provide new insight into mechanisms that couple DNA demethylation and transcriptional regulation. MeCP2, which has been considered a methyl-binding domain transcriptional repressor, was recently shown to function as a global transcriptional activator coupled to the AKT/mTOR pathway in mature neurons [148]. MeCP2 mutant neurons tend to be smaller and possess less RNA than their normal counterparts. After normalizing for differences on cell size based on their measurements, the authors reported that total amounts of RNA were reduced 1.2-fold in mutant neurons and that transcription in the mutant neurons is correspondingly reduced. These two findings change the framework for understanding how epigenetic changes impact normal cellular and neuronal function. As we move forward, it becomes increasingly clear that biological psychiatrists and neuroscientists need to reshape their thinking to adapt to constantly changing concepts that challenge conventional norms. It is only by applying new concepts and challenging new ideas that we will be able to understand how environmental stressors impact developing brain function and the origins of psychiatric diseases.

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Glossary

Bisulfite modification of DNA The genomic modification of DNA with bisulfite. Bisulfite modification of DNA was originally described as a means of positively identifying 5-methylcytosine residues on individual strands of DNA [60]. Genomic DNA following bisulfite modification and PCR would convert all Cs to Ts unless a specific position is methylated. The methyl group prevents deamination, leaving the cytosine moiety intact. The conversion of hydroxymethylcytosine to cytosine 5-methylenesulfonate was described some time ago and has been recently revisited [61]. HSO_3 (bisulfite) quickly converts 5-hydroxymethylcytosine to form cytosine-5-methylenesulfonate (CMS), which does not readily undergo deamination.

BiS-seq The genome-wide sequencing of DNA treated with bisulfite.

ChIP-seq Genome-wide sequencing of material generated following ChIP.

Chromatin immune precipitation (ChIP) A technique whereby DNA and chromatin proteins are cross-linked with formalin or another agent and sonicated. Antibodies specific to a particular transcription factor or modified histone are used to determine if the pulled-down sequences contain the gene of interest.

GABAergic Neurons using γ-Aminobutyric acid (GABA) as neurotransmitter.

Gene bodies Gene bodies are located downstream (3′) to transcription start sites, containing both exons and introns, and extending through the 3′ transcription termination point.

GLIB-seq Genome-wide sequencing method for detecting hydroxymethylcytosine [62]. The technique uses T4 β-glucosyltransferase (BGT) and uridine diphosphate glucose (UDP-glucose) to add a glucosyl group to protect hydroxymethylcytosines in genomic DNA. Sodium periodate is used to oxidize glycol groups to form aldehydes, which are then biotinylated and precipitated with streptavidin beads.

hMeDIP An immunopurification (5-hmC antibody) strategy to enrich sequences containing hydroxymethyl-CpG.

hMeDIP-seq A process where sonicated DNA fragments are precipitated and the enriched sequences are purified and sequenced using NGS.

Illumina® Infinium® HumanMethylation27 BeadChip™ array An earlier release of the Illumina Infinium methylation product line. It offers over 27,000 single site CpG probes corresponding to many of the highly regulated CpG sites in the human genome.

Illumina® Infinium® HumanMethylation450 BeadChip™ array Second-generation BeadChip produced by Illumina that contains over 480,000 probes, targeting over 99% of genes and 96% of CpG islands. Differences of 20% are detectable with 99% confidence [63].

MBD-seq Genome-wide sequencing method that uses the methyl-binding domain of MBD2 to enrich for CpG sequences bound by this domain.

MeDIP An immunopurification step that enriches for sequences based on the presence of a methylcytosine base.

MeDIP-seq Genome-wide sequencing of DNA fragments that have been immune precipitated using MeDIP.

Methyl C-seq Also called BiS-seq, this process is used for shotgun sequencing of BiS converted genomic DNA [64].

Next-generation sequencing (NGS) Refers to any number of massively parallel deep sequencing methods that generate short stretches of DNA sequence (shotgun libraries) that are then aligned to reference genomes or assembled using relatively high-speed computing [65].

OxBiS-seq A chemical method (potassium perruthenate, $KRuO_4$) to oxidize 5-hmC to 5-fC. Following BiS conversion, the 5-fC is converted to T while the original 5-mC remains a C. The technique can be used both genome wide and at specific loci [66].

Pyrosequencing A sequencing-by-synthesis method used to quantitatively determine the methylation of individual CG cytosines over short stretches of DNA (from 50 to 120 bp) [67].

Reduced representation bisulfite sequencing (RRBS-seq) A technique that first digests genomic DNA with restriction enzymes to enrich in CG-rich regions. Following gel purification, these DNA fragments are treated with BiS and sequenced. The technique is applicable to genome-wide sequencing following library preparation.

Sequenom MassARRAY High-throughput assay for evaluating methylation of bisulfite-treated DNA. It uses base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) introduced previously for SNP discovery [68].

TET1-assisted bisulfite sequencing (TAB-seq) 5-hmC groups are protected with B-GT and UDP-glucose forming 5-gmC. 5-mCs are then oxidized with recombinant mouse TET1 forming 5-caC. Following bisulfite modification, the protected 5-gmC groups remain as Cs while the 5caCs become Ts. The procedure can be used both genome wide and at specific loci [69].

CHAPTER

Pathogenic Histone Modifications in Schizophrenia are Targets for Therapy

11

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KEY CONCEPT

- Abnormal histone modifications present in patients with schizophrenia could be targets for drugs.

ABBREVIATIONS

BDNF Brain-derived neurotrophic factor
c-*fos* FBJ murine osteosarcoma viral oncogene homolog
CpG Cytosine–phosphate–guanine
CREB cAMP response element-binding protein
CRISPR Clustered regularly interspaced short palindromic repeats
CRYM Crystallin, mu
CYC1 Cytochrome C-1
DAO D-amino-acid oxidase
DAOA D-amino acid oxidase activator
DISC1 Disrupted in schizophrenia 1
DRD2 Dopamine type-2 receptor
DTNBP1 Dystrobrevin-binding protein 1
EGR1 Early growth response 1
GAD1 (GAD67) Glutamate decarboxylase 1 (brain, 67 kDa)
GRM2 Glutamate receptor, metabotropic 2
GWAS Genome-wide association scan
H2A Histone 2A

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HAT Histone acetyl transferase
HDAC Histone deacetylase
HDAC1 Histone deacetylase 1
HDM Histone demethyltransferase
HIST1H2BC Histone cluster 1, H2bc
HIST1H2BD Histone cluster 1, H2bd
HIST1H2BG Histone cluster 1, H2bg
HIST1H2BH Histone cluster 1, H2bh
HIST1H4K Histone cluster 1, H4k
HIST2H2BE Histone cluster 2, H2be
HMT Histone methyltransferase
HTR2A 5-Hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
KMT2A Lysine (K)-specific methyltransferase 2A
LSD1 Lysine (K)-specific demethylase 1A (KDM1A)
MDH Malate dehydrogenase
MeCP2 Methyl-CpG-binding protein 2
MLL1 Mixed-lineage leukemia 1
NEDD4 Neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase
NRG1 Neuregulin 1
NRSF RE1-silencing transcription factor
OAT Ornithine aminotransferase
PDE4B Phosphodiesterase 4B, cAMP-specific
RCOR1/2 REST co-repressor 1 and 2
REST RE1-silencing transcription factor
SIN3A SIN3 transcription regulator family member A
SIRT1 Sirtuin 1 (NAD-dependent deacetylase sirtuin-1)
SMARCA2 SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2
SUM01–4 Small ubiquitin-like modifier 1–4
SWI/SNF SWItch/Sucrose NonFermentable
SZ Schizophrenia
TALEN Transcription activator-like effector nuclease
TSA Trichostatin A
UBA6 Ubiquitin-like modifier activating enzyme 6
UFL1 UFM1-specific ligase 1

A brief introduction to histone modifications

In humans, each stretch of 146 base pairs of DNA with a negative electric charge is wrapped around a histone core with a positive electric charge enabling the compaction of 2 meters of DNA of each cell into 120 micrometers of chromosomes in an orderly manner. Four pairs of histone core proteins, including histone 2A and 2B (H2A and H2B), histone 3 (H3), and histone 4 (H4), assemble together and, along with the associated DNA, form the nucleosome, which is the basic unit of chromatin. While histone 1 (H1) as a linker element fastens the DNA at the entry and exit site to the nucleosome, approximately 50 base pairs of DNA link two neighboring nucleosomes to each other. This highly ordered structure not only protects DNA but also contributes to the regulation of gene expression. In fact, some of the amino acids of histone proteins could become acetylated/deacetylated, methylated/demethylated, phosphorylated/dephoshorylated, etc. (Figure 11.1), mediated by different types of enzymes [1]. These histone modifications could define the accessibility of a specific region of chromatin to transcription factors that regulate RNA synthesis. Histone modifications also define chromosomal conformations and looping configurations that determine the physical contact of promoter regions of the genes to far-away distal regulatory elements (e.g., enhancers, silencers) affecting gene expression in somatic cells [2].

In sperm, a smaller protein (protamine) replaces histone, allowing more condensation of the haploid chromosome to fit into the spermatozoid head [3]. The details of the reinstatement of histone modification patterns in paternal DNA after fertilization of the egg are not well understood. It has been proposed that the maternal chromosomes may introduce histone patterns to their equivalent paternal chromosome after fertilization. However, it is noteworthy to mention that most epigenetic marks, including DNA methylation, that may be involved in the regeneration of histone codes are erased in fertilized eggs. Recent experimental data presented evidence that the spermatozoid RNAs and a small fraction of the histone patterns maintained in the spermatozoid DNA may provide the basic memory for the regeneration of histone

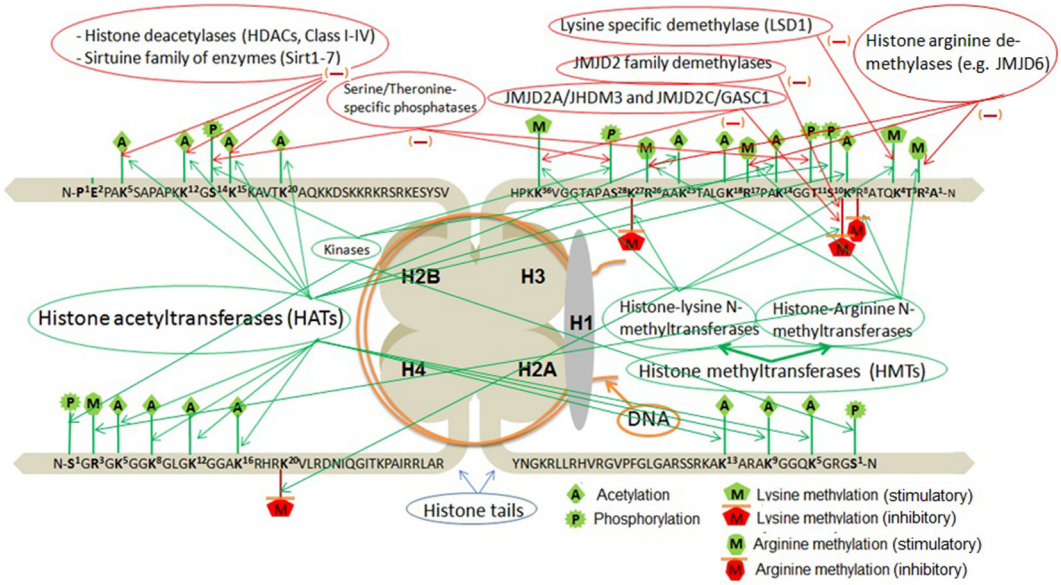

FIGURE 11.1

A schematic of the components of nucleosome structure which include histone 1 (H1), histone 2A (H2A), histone 2B (H2B), histone 3 (H3), histone 4 (H4), and the associated DNA. Various modifications of histones, carried out by different types of enzymes that add or remove [marked with arrows, where remove is marked with (–) sign] histone marks influence gene expression. The amino acids of histones are numbered from the N-terminal (N), and those subjected to acetylation (A), methylation (M), and phosphorylation (P) are in bold. In the amino acid sequence of histone tails, K, R, S, and T indicate lysine, arginine, serine, and threonine, respectively.

patterns [4,5]. Current knowledge also supports that several mechanisms, including DNA methylation, small and long non-coding RNAs, histone modification patterns [6–8] and the three-dimensional (3D) structure of genomic DNA [9], may cooperatively contribute in the recovery of epigenetic patterns after fertilization. Nevertheless, the underlying drivers of tissue specificity of DNA methylation and histone modifications that govern differentiation (while the DNA materials of all the cells of a complex organism are the same) remain unknown. Despite these caveats, as reviewed in this chapter, molecular studies in the last few decades have provided strong evidence that aberrant histone modifications are involved in the pathogenesis of schizophrenia (SZ) and that many drugs used in psychiatry, including antipsychotics, may normalize the aberrant histone modifications or modify the innate histone patterns that in turn may help to compensate other types of genetic/epigenetic dysfunctions in SZ.

Histone methylation and schizophrenia pathogenesis

Histones are common targets for mono-, bi-, or trimethylation affecting the binding affinity of the corresponding DNA to transcription factors and facilitating or inhibiting the corresponding gene expression in mature neurons [10,11]. For example, methylation of the fourth lysine of histone 3 (H3 lysine

4 monomethylation, H3K4^{me1}) and H3K4^{me3}, which are usually found at transcription start sites, and H3K36 ^{me3} located in the body of genes can facilitate the corresponding gene transcription [12–14]. On the other hand, while H3K9 ^{me3} at the promoter region is correlated with gene silencing, its presence in the gene body correlates with gene expression [15–17]. H3K27 ^{me1} is also linked to active genes, but H3K27 ^{me3} and H4K20 ^{me} are linked to gene silencing (Figure 11.1). Methylation of arginine 2, 17, and 26 of histone H3 and asymmetric dimethylation of arginine 3 of histone H4 (H4R3^{me2}) activate gene expression, but symmetric dimethylation suppresses gene expression [18,19]. Different types of histone methyltransferases (HMTs) or histone demethylases (HDMs) catalyze or remove, respectively, methyl groups from histones [10]. In general, gene promoter and enhancer regions contain higher proportions of H3K27 ^{me3} and H3K27 ^{me1}, respectively [20]. Their established status during differentiation along with other chromatin marks such as H3K9 ^{me3} and histone acetylation and DNA methylation define the cellular transcriptional profile in later developmental periods [21].

High-throughput chromatin immunoprecipitation analysis of the fetal brain with antibodies against H3K4^{me1}, as well as acetylated H3 at lysine 9 (H3K9^{ac}), which mark the enhancer and promoter region of genes, revealed multiple putative regulatory elements or core and proximal promoters for genes, such as *COMT*, *DAO*, *DAOA*, *DISC1*, *DTNBP1*, *NRG1*, and *PDE4B*, involved in SZ pathogenesis [22]. In other studies an increased baseline level of dimethylated lysine 9 of H3 (H3K9^{me2}), which is a repressive chromatin mark, was observed in lymphocytes as well as in postmortem brain samples of SZ patients compared with control subjects. The higher levels of H3K9^{me2} have also been associated with a younger age of disease onset [23,24].

Although there is a progressive age-dependent increase of H3K4^{me} at the GAD1 promoter and other GABAergic genes, a decrease in H3K4^{me} at the GAD1 promoter associated with a decrease in *GAD1* expression has been reported in the prefrontal cortex of SZ patients. The decline in H3K4^{me} was attributed to the dysfunction of mixed-lineage leukemia 1 gene (*MLL1* or *KMT2A*) acting as an HMT in GABAergic and other neurons of the cerebral cortex [25]. Notably, a subsequent animal study found that H3K4^{me} is increased by clozapine in the mouse cerebral cortex [25]. Recent findings also indicate that higher-order chromatin structures that delineate chromosomal looping and modulate physical contact of promoter regions of genes to *cis*-acting enhancers or silencers are altered at the GAD1 promoter region in the frontal cortex of some patients with SZ and play a role in regulating *GAD1* expression [26].

Aberrant histone acetylation in schizophrenia

The lysine amino acid of various types of histone proteins is subjected to dynamic acetylation or deacetylation that define the chromatin state of specific genes and thus their transcription status. H3 lysine 9 acetylation (H3K9^{ac}) catalyzed by histone acetyl transferases (HATs) that correspond to unmethylated promoter DNA facilitate the corresponding gene transcription. Acetylation of other lysine residues that are generally linked to gene expression include K14, K18, K23, K36, K53 of histone H3; K5, K9, K13 of histone H2A; K5, K12, K15, K20 of histone H2B; and K5, K8, K12, K16 of histone H4 (Figure 11.1). On the other hand, removal of the acetyl group from histones mediated by histone deacetylases (HDACs) inhibits gene transcription [1,19]. The latter effect can be reversed by different drugs such as sodium butyrate and valproate that non-specifically inhibit HDACs, increasing the expression of genes regulated by the modification of histone patterns [1,12,13]. There is also increasing evidence that environmental cues modify histone acetylation. For instance, the stress of chronic social defeat increases

H3K9 and H4K5/8/12/16 acetylation in the dorsal raphe nucleus, and H3K18 acetylation in the medial prefrontal cortex of rats that were less resilient to stress compared with controls [27]. However, a decrease in H4K8 acetylation was observed in both resilient and non-resilient rats compared with control rats. Additionally, there were changes in the expression of *SIRT1* and *SIRT2* (NAD-dependent deacetylases) in the ventral hippocampus, and *Kat5* (K[lysine] acetyltransferase 5) in the medial prefrontal cortex of these animals [27]. These observations are consistent with the notion that histone modifications could contribute to the underlying mechanisms enabling adaptation to environmental conditions.

One of the earliest reports of SZ was related to an altered chromatin ultrastructure in neutrophils of patients compared with control subjects [28]. Interestingly, pimozide, which is a highly potent antagonist for the dopamine type-2 receptor (DRD2) was found to attenuate the increased arginine-rich histones in SZ patients [29]. Despite these initial reports, there was a long gap in studies in the field for decades. With the advent of new techniques, gene expression profiling revealed aberrant expression of histone deacetylase 3 (HDAC3) in the temporal cortex [30] and dysregulation of many other histone-related genes, such as *HIST1H2BD*, *HIST1H2BC*, *HIST1H2BH*, *HIST1H2BG*, *HIST1H4K*, and *HIST2H2BE*, in the blood of SZ patients [31,32]. Other investigators have also reported an increased level of H3-(methyl)arginine 17 associated with decreased expression of several genes, including *CRYM*, *CYTOC/CYC1*, *MDH*, and *OAT*, which are among the metabolic genes in the prefrontal cortex of a subset (~20%) of SZ patients [33]. Additionally, analysis of the results from a genome-wide association scan (GWAS) in SZ also revealed key roles for genes, related to the SWI/SNF chromatin-remodeling complex which governs dendritic growth during SZ pathogenesis [34]. In mammals, this complex includes either SMARCA2 or SMARCA4, which forms a super-complex with several other gene products such as REST/NRSF, SIN3A/B, RCOR1/2, HDAC1/2, and MeCP2 that bind to methylated CpGs of DNA, influencing gene expression [34].

An increased expression of HDAC1 in the frontal cortex of SZ patients was also reported from the analysis of the National Brain Databank microarray collection (Harvard Brain Tissue Resource Center). In addition, expression levels of HDAC1, HDAC3, and HDAC4 were associated with a reduced mRNA expression of *GAD1*, a gene known to be regulated by epigenetic modifications and involved in the pathogenesis of SZ [35]. These results are consistent with similar observations made in the hippocampus of SZ patients [36]. Furthermore, recent studies in mice expressing adenoviral HDAC1 transgene in the prefrontal cortex also uncovered deficits in working memory, abnormality in locomotor response, and repetitive behaviors associated with downregulation of genes related to SZ pathogenesis [37]. In summary, considering the lack of opportunity for brain biopsy in humans, these findings support the importance of studies performed with the postmortem brain samples.

Other types of histone modifications associated with SZ pathogenesis

Phosphorylation, ubiquitylation, sumoylation, and NEDDylation are among other common modes of histone modification that are linked to activation or suppression of gene expression. For example, phosphorylation of threonine 3 or 11 of histone H3 (H3T3p or H3T11p) and serine 10 or 28 of histone H3 (H3S10p or H3S28p) and the linker histone H1, as well as S14 of histone H3B and S1 of histone H2A and H4, have been linked to transcriptional activation (Figure 11.1). Although sumoylation, mediated by SUMO1–4 (small ubiquitin-like modifier 1–4) which covalently conjugate to lysine residues of their substrates, is linked to gene suppression, ubiquitylation could have a dual role. For example,

ubiquitylation of K2B123 and K2A119 (where K indicates lysine residue) are linked to gene activation and suppression, respectively [12,19].

Aberrant ubiquitination, Ufmylation, SUMOylation, and NEDD8ylation have been reported in postmortem brain tissues dissected from the superior temporal gyrus of SZ patients [38]. These aberrations include an increase in K63 polyubiquitination, but decreases in protein ubiquitination, free ubiquitin, K48-linked ubiquitination, UBA6 (an ubiquitin E1 activase), NEDD4 (an E3 ligase), UFL1 (an E3 ligase involved in ufmylation), and PIAS3 (involved in SUMOylation). NEDD8ylation was also found to be dysregulated in SZ, with decreased levels of UBA3 (an E1 activase) and Rnf7, which is classified as E3 ligase [38]. This area of research is at a very early stage with reference to SZ and requires more studies in the future to uncover its potential impact on disease pathogenesis.

Current therapeutic targets modulating histone codes

Based on several studies, valproate, being a non-specific HDAC inhibitor, induces a dose-dependent increase in acetylated H3 of lymphocytes in SZ, and in particular in patients with bipolar disorder [39]. Interestingly, in cell culture experiments, the lymphocytes of SZ patients were less sensitive to treatment with trichostatin A (TSA), another non-specific HDAC inhibitor, compared with non-SZ individuals, suggesting that in SZ, the chromatin state might be more resistant to drug treatment [40]. The report of the same investigators also indicated that in human lymphocytes valproate and TSA could increase *GAD67* mRNA fourfold, with corresponding two- and five-fold increases in total H3K9,K14 acetylation and GAD1 promoter H3K9,K14 acetylation, respectively. Interestingly, in patients with bipolar disorder, higher baseline levels of H3K9 and H3K14 acetylation were observed compared with SZ patients, but there was a higher level of *GAD67* (*GAD1*) expression in the lymphocytes of subjects harboring therapeutic levels (>65 μg/mL) of valproate [23]. Notably, some of the antipsychotic drugs could also affect the histone codes. For example, MS-275, a benzamide with HDAC inhibitory effects, could increase H3 acetylation of *GAD1* and *RELN* in the frontal cortex and hippocampus but not in striatum of mice [41]. In addition, clozapine and sulpiride (but not haloperidol or olanzapine) facilitated valproate-induced chromatin remodeling [42]. A recent study also reported that chronic use of atypical antipsychotic drugs (but not haloperidol) was associated with an increased expression of HDAC2 (but not HDAC1 and HDAC4) and its binding to GRM2 promoter, thus decreasing histone acetylation and *GRM2* expression in mice and in the frontal cortex of SZ patients. This effect was mediated by HTR2A (serotonin type-2 receptor), thus it was abolished in *HT2RA* null mutant mice. The use of HDAC inhibitors could also abolish this effect in mice, attenuating the effects of atypical antipsychotic drugs [43]. In a subsequent study, the same investigators noted that a disrupted HTR2A-dependent signaling resulted in the loss of *Egr1* (early growth response 1) promoter activation, which may mediate a decrease in histone H3 and H4 acetylation and an increase in the level of $H3K27^{me3}$ at the GRM2 promoter leading to its suppression [44]. However, a different report suggested that clozapine-induced chromatin remodeling of other gene promoters (such as GAD1 and RELN) can be potentiated by valproate, which is independent of catecholamine or serotonin receptors [45]. Additionally, lurasidone, a new atypical antipsychotic drug, also increased the expression of HDAC1, HDAC2, and HDAC5 that could be augmented by valproate. However, concomitant use of these drugs could increase brain-derived neurotrophic factor (*BDNF*) expression more than either drug alone in the ventral hippocampus [46].

In addition to antipsychotic drugs, antidepressant drugs that are also used concomitantly in SZ patients are epigenetic modifiers. For example, amitriptyline inhibits the activity of HDACs and DNMTs, thus increasing H3 acetylation and DNA demethylation [47]. Imipramine inhibits HDAC5 and increases histone acetylation of *BDNF III and IV* promoters [48]. Fluoxetine can reverse the stress-induced reduction of H3 acetylation and $H3K9^{me3}$ in rat hippocampus and dentate gyrus, respectively [49]. Tranylcypromine inhibits *LSD1* (lysine-specific demethylase 1), which demethylates $H3K4^{me1}$, $H3K4^{me2}$, $H3K9^{me1}$, and $H3K9^{me2}$, resulting in the transcription induction of many genes, including *Egr1* [50]. Lithium also increases H3 acetylation and phosphoacetylation in the central nucleus of the rat amygdala, which can be potentiated by HDAC inhibitors [51].

Interestingly, other animal studies found that electroconvulsive therapy could also alter the levels of histone acetylation in the promoter regions of *BDNF III/IV*, c-*fos*, and *CREB* with a corresponding increase in the expression of these genes [52]. In summary, the current experimental evidence suggests that, while histone modifications may contribute to the pathogenesis of SZ, some of the commonly used drugs in psychiatry and even electroconvulsive therapy may also act by targeting the altered histone codes in SZ.

Methodological challenges of histone modification analyses

Despite significant progress that has been made with histone modification analyses and in generating large amounts of data relevant to SZ pathogenesis in the last few decades, lack of consistency in the results from different studies remains a dilemma that cannot be addressed in the short term. The dynamicity and complexity of tissue-specific epigenetic patterns and methodological differences are among the main causes of inconsistency and irreproducibility in the reported alterations. Most of the methods currently used in chromatin modification analysis lack sufficient sensitivity and specificity. For example, most of the methods related to the analysis of histone acetylation and methylation marks are reliant on chromatin immunoprecipitation following sonication or enzymatic restriction of genomic DNA. Sonication randomly breaks the genomic DNA into different size fragments (optimally 200–1000 bp) but many factors such as preparation of tissue homogenate, volume, and location of the sonication probe in sample tubes as well as foam formation may affect the efficiency of sonication. Enzymatic restriction also may not cover all of the regions of interest. Although the approximate sizes of fragmented DNA samples are evaluated in agarose gel electrophoresis to assess if the fragment sizes are approximately 200–1000 bp, this evaluation process is semiquantitative at best. Because most of the antibodies for the immunoprecipitation of particular histone marks are not specific, altogether the current techniques cannot detect minimal differences (~%25) in histone acetylation and/or methylation between cases and control subjects, even though subsequent real-time or microarray analysis might be more efficient and accurate. Furthermore, as epigenetic marks are tissue and cell specific and the brain, as the main affected organ in SZ, is not homogeneous, the detection of epigenetic changes in specific neuronal cells is not possible with most of the techniques that have been used in the studies reviewed here. In fact, the brain consists of two component parts at a glance, the white and gray matter, and the gray matter consists of six different layers with different functional and epigenetic characteristics. Assuming that one of the layers of the gray matter loses the normal landscape of histone patterns in one of the chromosome pairs ($1/6 \times 1/2 = 1/12$ of the gray matter), this could have significant functional impact in that cortical layer. However, almost none of the current techniques can detect this level of epigenetic alteration in the aggregate of the gray matter layers. Although laser microdissection can help

to collect limited numbers of specific cells for other types of epigenetic analysis (e.g., DNA methylation, microRNA), this approach has not been able to provide the threshold of specimen required for the histone modification analyses. Therefore, the development of new techniques remains a necessity to map out the histone modification landscapes of different neuronal cells affected in SZ.

Conclusion and perspectives

The three-dimensional architecture of chromatin dictated by histone modifications at the regulatory region of genes involved in SZ pathogenesis is becoming increasingly important for deciphering therapeutic strategies. The progress in cataloging the various histone modifications in SZ and the effects of commonly used drugs suggest that, although some drugs already target pathologic modifications, improved success in treating patients could be achieved through the use of a cocktail of drugs directed at multiple disease-specific epigenetic alterations. While non-specificity of current psychiatric drugs, as well as other drugs in medicine, remains a dilemma, the development of novel techniques using transcription activator-like effector nuclease (TALEN) or clustered regularly interspaced short palindromic repeats (CRISPR) may help to deliver epigenetic drugs to specific DNA sequences for modification of the affected DNA or histone patterns in the coming years. In order to deliver these drugs to affected neuronal pathways, concurrent activation of these pathways by transcranial magnetic stimulation, or other means that could increase cellular metabolism and transport of different molecules (e.g., glucose), may help to deliver epigenetic drugs attached to these molecules to the activated cells at a much higher rate compared with other cells. However, the use of these therapeutic remedies is largely dependent upon the identification of key pathways and epigenetic aberrations that lead to the disease phenotype, an issue that, despite recent exponential progress using advanced existing technologies, requires a much more concerted effort to accomplish.

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Glossary

Sonication The process of dispersing, disrupting, or inactivating particles in a sample using sound-wave energy.

CHAPTER

Non-Coding RNAs and Psychiatric Disorders

12

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KEY CONCEPT

- There is accumulating evidence that non-coding RNAs, especially microRNAs (miRNAs), are involved in the pathogenesis of psychiatric disorders.

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ABBREVIATIONS

BDNF Brain-derived neurotrophic factor gene
DGCR8 Di-george critical region 8 gene
DISC1 Disrupted in schizophrenia 1 gene
DLPFC Dorsolateral/prefrontal cortex
DRD1 Dopamine receptor 1 gene
EGR3 Early growth response 3 gene
eQTLs Expression quantitative trait loci
ERBB4 v-erb-A avian erythroblastic leukemia viral oncogene homolog-like 4 gene
GRIA2 Ionotropic AMPA glutamate receptor subunit 2 gene
GWAS Genome-wide association study
lncRNA Long non-coding RNA
miRNA microRNA
ncRNA Non-coding RNA
NRG1 Neuregulin 1 gene
ORF Open reading frame
PBMCs Peripheral blood mononuclear cells
PFC Prefrontal cortex
piRNA PIWI-interacting RNA
QK1 Quaking homolog, KH domain gene
RT-PCR Reverse transcription–polymerase chain reaction
SAT1 Spermidine/spermine N^1-acetyltransferase1 gene
siRNA Small (short) interfering RNA
SMOX Spermine oxidase gene
snoRNA Small nucleolar RNA
SRSF1 Serine/arginine-rich splicing factor 1 gene
STG Superior temporal gyrus
VSNL1 Visinin-like 1 gene

Introduction

The human genome is comprised of about 3 billion base pairs; however, there are only about 20,000–25,000 protein-coding genes in the human genome [1]. The total length covered by the coding exons is about 1.2% of the genome; the untranslated regions of the transcripts is estimated to cover approximately another 0.7% of the entire genome [1]. Although previously it was thought that most of the human genome excluding the protein coding genes was simply structural in nature, it is now known that this part of the human genome can be extensively transcribed into non-coding RNAs (ncRNAs). ncRNAs are RNAs that are transcribed from DNA but are not translated into proteins [2]. Genes encoding ncRNAs produce a functional RNA product rather than a translated protein [3], and many are involved in the processing and regulation of other RNAs such as messenger RNA, transfer RNA, and ribosomal RNA [2]. ncRNAs are comprised of transcripts that contain a high density of stop codons and do not have any clear open reading frame (ORF), a stretch of DNA that begins with an initiation codon and ends with a nonsense codon [4]. All ORFs have the potential to encode a protein or a polypeptide; however, ncRNAs are very difficult to predict from genomic sequences [5]. It has been predicted that there are more than 4000 ncRNAs in humans, with many of these likely reflecting pseudogenes and repeat-derived sequences [3]. A useful ncRNA resource available on the web is Rfam, which is a comprehensive database of ncRNA families that allows users to view and download multiple sequence alignments, read annotations, access literature, and visit other RNA databases [6].

Types and functions of non-coding RNAs

Although there are no clear-cut differences between different classes of ncRNAs, these molecules can be divided into three classes [7,8]. One class of ncRNAs is short ncRNA, including microRNA (miRNA), small (or short) interfering RNA (siRNA), and PIWI-interacting RNA (piRNA). These

ncRNAs are short nucleotides, the most studied of which are miRNAs. miRNAs are 21–23 nucleotides in length, and in humans more than 2000 mature miRNA sequences have been reported [9]. In animals, miRNAs mediate posttranscriptional gene silencing by inhibiting the translation of messenger RNA into proteins. The translation of proteins may be inhibited in two ways: by messenger RNA degradation or by inhibiting translation initiation [7]. miRNAs are estimated to regulate the translation of more than 60% of protein-coding genes and are involved in controlling several processes, including cell proliferation, differentiation, apoptosis, and development. Although some miRNAs regulate specific individual targets, others can act as master regulators of a cellular process. Hence, key miRNAs control the expression of hundreds of genes at the same time, and many miRNAs regulate their targets cooperatively [7]. Small interfering RNAs are 20–25 nucleotides in length and are involved in gene regulation, transposon control, and viral defence. piRNAs are 27–30 nucleotides long and are mainly restricted to the germ line. They are involved in the regulation of transposon activity and chromatin states.

Another class of ncRNAs is medium ncRNA, including small nucleolar RNAs (snoRNAs), which are 60–300 nucleotides long. snoRNAs are involved in the regulation of methylation, and pseudouridylation of ribosomal RNA and could also be involved in gene regulation. The third class of ncRNAs is long non-coding RNA (lncRNA). lncRNAs are more than 200 nucleotides long, and there are over 1000 known lncRNAs in humans. They are widespread throughout the genome; indeed, they make up the largest portion of the human non-coding transcriptome. They are involved in the epigenetic modification of DNA by recruiting chromatin remodeling complexes to specific loci, in physiological processes like X-chromosome inactivation, and genomic imprinting. More details of ncRNAs are given in Table 12.1.

Table 12.1 Types of Non-Coding RNAs

Non-Coding RNA	Abbreviation	Length	Description
Short Non-Coding RNA			
microRNA	miRNA	21–23 nt	Single-stranded RNA folds into double-stranded RNA structure. After processing and binding to RNA-induced silencing complex (RISC), miRNAs target messenger RNAs to regulate translation.
Small (short) interfering RNA	siRNA	20–25 nt	Regulate a specific gene using complementary sequence. Posttranscriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) pathways are present.
PIWI-interfering RNA	piRNA	27–30 nt	Interact with PIWI proteins for chromatin regulation and transposon silencing.
Medium Non-Coding RNA			
Small nucleolar RNA	snoRNA	60–300 nt	Involved in modifications of ribosomal RNA.
Long Non-Coding RNA			
Long non-coding RNA	lncRNA	>200 nt	Many have specific targets and are critical for X-chromosome inactivation in mammals, meiotic sex chromosome inactivation, and *Hox* gene regulation.

Source: Adapted with permission from Collins et al. The epigenetics of non-coding RNA. In: Tollefsbol T, editor. Handbook of epigenetics: the new molecular and medical genetics. Oxford: Elsevier; 2011. p. 49–61.

BOX 12.1 SOME EPIGENETIC EFFECTS OF NON-CODING RNAs

- Regulation of DNA methylation
- Modification of chromatin
- Chromatin remodeling
- X-chromosome inactivation
- Genomic imprinting.

It is known that increasingly large non-coding sequences of DNA make up the genomes of organisms with correspondingly greater levels of developmental complexity [10,11]. It has been proposed that an increase in the types and numbers of ncRNAs, especially miRNAs, which has occurred continuously during vertebrate evolution, was responsible for the unfolding of vertebrate complexity, especially in the brain [12]. ncRNAs are also involved in the functioning of all aspects of cell biology. Functions of ncRNAs in normal cell physiology include the following: X-chromosome inactivation, maintenance of chromatin structure, transcription, cell stress, T-lymphocyte activation, neural function and differentiation, and embryonic development [5]. In addition, ncRNAs are involved in epigenetic mechanisms of gene expression. Indeed, ncRNAs, especially small ncRNAs, are now known to be involved in a variety of epigenetic mechanisms [5,12–14]: regulation of DNA methylation, modification of chromatin, chromatin remodeling, X-chromosome inactivation, and genomic imprinting. Some of the epigenetic effects of ncRNAs are listed in Box 12.1. ncRNAs have also been implicated in the pathogenesis of several complex diseases such as cancer, neurological diseases such as Parkinson's disease and prion diseases, cardiovascular diseases such as heart failure and cardiac arrhythmias, rheumatoid arthritis, and psychiatric disorders [7,8].

Role of non-coding RNAs in the brain

The role of various types of ncRNAs in the brain has been studied; however, most work on ncRNAs in the functioning of the brain has focused on miRNAs [15–18]. ncRNAs were thought to have played a major role in the evolution of the human brain [15], due to their ability to couple sequence-specific (digital) and conformational (analog) information in the same molecule; they could lower the bioenergetic costs of information processing, participate in accelerated and mainly unconstrained evolutionary transitions, serve as sensitive and rapidly reversible biosensors of both environmental and interoceptive cues, and could readily adapt to intricate, multilayered, and challenging environmental conditions. These unique properties of ncRNAs are thought to have allowed them to promote complex DNA/RNA/protein interactions and dynamic crosstalk between traditional protein-based signal transduction cascades and the more malleable properties of the genomic architecture. This would have been facilitated with the advent of RNA-binding proteins, which resulted in an increase in the temporal and spatial regulation of gene expression and function. Another feature of ncRNAs that would have helped contribute to human brain evolution is their ability to function as molecular adaptors, such that they could assume a great number of analog shapes at a fraction of the bioenergetic cost of proteins, which have a more limited conformational repertoire. Moreover, within the brain, ncRNAs participate in elaborate activity-dependent temporal and spatial control of gene expression

BOX 12.2 SOME FUNCTIONS OF NON-CODING RNAs IN THE BRAIN

- Neurogenesis
- Neural development
- Cellular responses leading to changes in synaptic plasticity
- Learning and memory
- Circadian clock
- Social behaviors
- Response to stress.

and function. This occurs through the dynamic repression, activation, and sequestration of diverse ncRNAs during bidirectional axodendritic transport in association with RNA-binding proteins contained in neuronal granules and at synaptic terminals [19].

ncRNAS, especially miRNAs, are also known to play essential roles in every aspect of the functioning of the brain, including neurogenesis, neural development, and cellular responses leading to changes in synaptic plasticity [18]. ncRNAs are also involved in learning and memory [16], the circadian clock [16], and social behaviors [16]. In rodents, they have been shown to take part in the behavioral vulnerability to early-life stress [20] as well as repeated restraint stress [21]. Some of the functions of ncRNAs in the brain are listed in Box 12.2. In humans, ncRNAs have been implicated in the pathogenesis of disorders of the brain [15]. This chapter discusses the current state of knowledge regarding the role of ncRNAs in the pathogenesis of psychiatric disorders. Rogaev [22] hypothesized as early as 2005 that ncRNAs could be involved in the pathogenesis of psychiatric disorders such as schizophrenia. His hypothesis was based on the following facts: (1) Small ncRNAs such as miRNAs are abundant in the brain and there are brain-specific and brain-enriched miRNAs; (2) In non-mammalian models, miRNAs have been shown to be important for the development of the nervous system; (3) It had been shown that the activity of miRNAs changed and was regulated during the development of the brain; (4) miRNAs were known to be involved in neurogenesis. Experimental work done subsequently appears to support Rogaev's hypothesis, as discussed below.

Role of non-coding RNAs in schizophrenia

Schizophrenia is the psychiatric disorder that has been most extensively investigated with respect to ncRNAs, and most studies on the role of ncRNAs in psychiatric disorders including schizophrenia have focused on miRNAs. Such studies have involved postmortem samples of brains of schizophrenia patients, cerebrospinal fluid samples, and peripheral blood samples [23]. Dysregulation of miRNAs in schizophrenia and other psychiatric disorders is thought to be due to altered expression or changes to the targets of miRNAs. Due to deletions, amplifications, or single-nucleotide polymorphisms in miRNAs, there can be overexpression or underexpression of the miRNAs with ensuing changes to their targets. Alternatively, changes in the miRNA processing machinery or single nucleotide polymorphisms in the target region for a miRNA can lead to inappropriate silencing of messenger RNAs [23].

Studies on postmortem brain

The first study to investigate abnormalities of ncRNAs in schizophrenia was that of Perkins and colleagues in 2007 [24]. Due to the important role of miRNAs in regulating brain development early in life and in mediating synaptic plasticity later in life, these authors hypothesized that schizophrenia may be associated with altered miRNA profiles. They compared the expression of 264 human miRNAs in postmortem prefrontal cortex (PFC) tissue of patients with schizophrenia or schizoaffective disorder with that in psychiatrically unaffected subjects using a custom miRNA microarray. They found that 16 miRNAs were differentially expressed in the PFC of the patients, with 15 expressed at lower levels and 1 at a higher level. The expression of 12 selected miRNAs was also investigated by quantitative reverse transcription–polymerase chain reaction (RT-PCR). For the eight miRNAs that were found to be underexpressed by microarrays in schizophrenia samples in comparison with control samples, seven were also underexpressed with quantitative RT-PCR. A little later, Beveridge et al. [25] analyzed global miRNA expression in postmortem cortical gray matter from the superior temporal gyrus (STG) of patients with schizophrenia and found significant upregulation of miR-181b expression. This finding was supported by quantitative RT-PCR analysis of miRNA expression in a cohort of matched pairs of schizophrenia patients and non-psychiatric controls. This finding is potentially of interest and significance because it has been shown that there is enrichment of the expression of miR-181b in the brain, involving both neurons and neuroglia. Moreover, miR-181b is known to regulate the expression of several target genes with potential significance to the pathogenesis of schizophrenia, such as the calcium sensor gene visinin-like 1 (*VSNL1*) and the ionotropic AMPA glutamate receptor subunit 2 gene (*GRIA2*). The messenger RNAs for these genes were also found to be downregulated in the same cortical tissue in the patients with schizophrenia. The same research group later followed up on this work by investigating miRNA expression in postmortem STG and dorsolateral/prefrontal cortex (DLPFC) from patients with schizophrenia [26]. It was found that in both of the examined areas of the brain there was a significant increase in global miRNA expression, and that this was associated with an increase in primary miRNA processing and an increase in the microprocessor component *DGCR8* messenger RNA in both the STG and the DLPFC. *DGCR8* is involved in the biogenesis of miRNAs. The authors found that 59 miRNAs were upregulated in the STG of patients with schizophrenia (21% of the miRNAs expressed in this region of the human brain). In the DLPFC, the authors found that 26 miRNAs were upregulated (9.5% of the miRNAs expressed in this area of the brain). The authors suggested that their findings could be important for the understanding of schizophrenia because the miRNAs found to be overexpressed could be involved in controlling cortical structure and neural plasticity. The above studies have been followed by several other studies investigating the role of miRNAs in postmortem brain tissues of patients with schizophrenia [27–35]. These studies found altered expression of several miRNAs, most of them showing decreased expression of miRNAs [27,30–32,34], although some also showed increased expression of miRNAs [28,29,33,35].

As referred to above, several studies have investigated the role of miRNAs in postmortem samples of brain, but the findings of these studies conducted so far are not consistent [23]. Possible reasons for the inconsistencies include different methodologies of studies, with some investigators focusing on one or two miRNAs and others on hundreds of miRNAs; differences in sample sizes used between studies; different areas of the brain being investigated by the different studies; the effects of gender

differences between studies; and the effects of psychopharmacological drugs such as antipsychotics on the expression of miRNAs [23]. There are also many miRNAs that are consistently reported to show significant alterations in postmortem brain samples, but in opposite directions [36]. Some miRNAs show consistent alterations in studies of postmortem brain but are altered in the opposite direction in studies on peripheral tissues [36]. However, some miRNAs have been repeatedly identified to be abnormally altered across many studies of schizophrenia [23]. Such miRNAs include the miR-15 family (which includes miR-195 and the closely related miR-107) that were upregulated in the frontal cortex. These miRNAs target genes implicated in schizophrenia such as the brain-derived neurotrophic factor gene (*BDNF*), the dopamine receptor 1 gene (*DRD1*), neuregulin 1 (*NRG1*), and the early growth response 3 gene (*EGR3*).

There is increasing evidence that lncRNAs also play a key role in the development and functioning of the brain. lncRNAs are thought to be involved in the generation of an enormous array of regional neuronal and glial cell subtypes that are present in the brain [37]. They are also thought to be involved in other brain functions such as homeostasis, stress responses, synaptic plasticity, and promoting long-term changes in synaptic strength [37]. However, although lncRNAs are a very abundant type of ncRNA and are highly expressed in the brain, they have been relatively poorly characterized and their roles in the brain have not been studied in detail. Nevertheless, Barry and colleagues [38] recently showed that lncRNAs are dynamically regulated by neuronal activation and hence are likely to regulate activity-dependent neuronal processes. The authors also found downregulation in the STG of Gomafu, an lncRNA that has been shown to be involved in brain and retinal development. The authors also showed that Gomafu binds directly to the splicing factors *QK1* and *SRSF1* and that dysregulation of Gomafu leads to alternative splicing patterns that resemble those observed in schizophrenia for the schizophrenia-associated genes *DISC1* and *ERBB4*. The authors also showed, for the first time, that Gomafu binds to QK1, a protein implicated in the pathogenesis of schizophrenia, suggesting that Gomafu could contribute to the pathogenesis of schizophrenia.

Studies on peripheral tissues

In addition to postmortem brain samples, several studies have investigated miRNAs in peripheral blood samples [39–42], cerebrospinal fluid [43], and olfactory neuroepithelium [44] of patients with schizophrenia. The studies on peripheral blood samples included mononuclear leukocytes [39,40], serum [41], and plasma [42]. Because peripheral tissues, unlike the brain, are readily accessible, they have the advantage that they may be the source of biomarkers for the diagnosis of schizophrenia and other psychiatric disorders [45,46]. In a recent multicenter study by Keller and colleagues [47] the expression profiles of 863 miRNAs were assessed by array analysis of 454 blood samples from humans with different types of cancers and non-cancer diseases, as well as from unaffected individuals, who served as controls. This miRNome was validated by quantitative RT-PCR. It was found that blood cells have a miRNA pattern that varies between different diseases, and there were consistently abnormal profiles for all the diseases that were tested. Moreover, pathway analyses confirmed disease association of the respective miRNAs. The authors suggested that their work provides support for the feasibility of using miRNA expression patterns in peripheral blood cells as the basis for detection of disease.

Role of non-coding RNAs in bipolar disorder

Studies on postmortem brain

Compared with schizophrenia, relatively few studies have investigated abnormalities of ncRNAs in the postmortem brain from patients with bipolar disorder [28,30,33–35]. Similarly to schizophrenia, though, several miRNAs have been shown to be dysregulated in postmortem brain samples of patients with bipolar disorder. Studies have shown both an upregulation [33,35] and a downregulation [30,34] of miRNAs, or a combination of both [28]. There is evidence from studies of miRNAs in patients with bipolar disorder to suggest that there could be a partially shared molecular basis for the development of bipolar disorder and schizophrenia [23].

Studies on peripheral tissues

Even less work has been done on the role of miRNAs in peripheral tissues of patients with bipolar disorder than on postmortem brain samples. Rong et al. [48] investigated plasma levels of miR-134 in patients with the manic phase of bipolar disorder before and after treatment with mood-stabilizing drugs. The authors focused on miR-134, as this miRNA had been identified as a potential regulator of dendritic spine volume and synapse formation. They found that plasma levels of this miRNA in drug-free patients were significantly lower than those of controls and that the miRNA levels in patients increased after starting treatment with mood-stabilizing drugs. The authors suggested that miRNA-134 could serve as a potential biomarker for bipolar disorder.

Role of non-coding RNAs in major depressive disorder

Just as in schizophrenia and bipolar disorder, the role of ncRNAs in major depressive disorder is also being actively investigated, although to date the number of studies conducted has been relatively small and such studies have focused on miRNAs [49,50].

Studies on postmortem brain

Smalheiser and colleagues [51] investigated whether miRNAs are altered in the PFC of antidepressant-free depressed suicide subjects in comparison with well-matched non-psychiatric controls. The authors found that overall miRNA expression was significantly and globally decreased in the PFC of the depressed patients. They inferred from their results that widespread changes in miRNA expression are likely to contribute to the pathogenesis of major depression and/or suicide. Another study on miRNAs in postmortem brain of patients with major depression was conducted by Lopez et al. [52]. This group had previously shown that the expression of the polyamine genes *SAT1* and *SMOX* was downregulated in the brain in suicide completers. In this light, they hypothesized that the dysregulation of these genes could be influenced by miRNAs. Hence, the authors identified many miRNAs that target the 3′-UTR of *SAT1* and *SMOX*. They profiled the expression of 10 miRNAs in the PFC of depressed suicide completers in comparison with psychiatrically healthy control subjects. They found that many miRNAs were significantly upregulated in the PFC of the depressed suicide completers in comparison with the control subjects. In addition, the authors demonstrated a significant correlation between these miRNAs and the expression levels of both *SAT1* and *SMOX*.

Studies on peripheral tissues

The role of miRNAs in major depressive disorder in peripheral tissues has also been investigated. Belzeaux and colleagues [53] compared, in peripheral blood mononuclear cells (PBMCs), miRNA expression between patients with major depression and psychiatrically normal controls at baseline and also after 8 weeks of antidepressant treatment. Of the 762 miRNAs assayed, about one-third showed mean expression values above the background level for detectability, and these were further analyzed. It was found that the expression of 14 miRNAs was dysregulated at baseline and after 8 weeks of antidepressant treatment, with 9 being upregulated and 5 being downregulated. Only two miRNAs showed stable overexpression in depressed patients during the 8-week study period compared with the controls. During the study period, some miRNAs showed significant variations of expression among the treated patients, with seven being upregulated and one being downregulated. The authors suggested that their results confirm the potential utility of miRNA signatures as markers of changes in the clinical status of patients with major depression. More recently, Li et al. [54] explored the effects of the miRNAs miR-132 and miR-182 on serum levels of BDNF in patients with major depression. Compared with healthy controls, the depressed patients had lower serum levels of BDNF and higher serum levels of miR-132 and miR-182. The authors also found a significant negative correlation between levels of depression among the patients and serum BDNF levels, and a significant positive correlation between levels of depression among the patients and miR-132 levels. In addition, there was a significant inverse correlation between serum BDNF levels and miR-132/miR-182 levels in depressed patients.

Polymorphisms of genes encoding miRNAs associated with psychiatric disorders

Genome-wide association studies (GWASs) and expression quantitative trait loci (eQTLs) are being used to identify genetic variants associated with disease risk and gene expression. Certain polymorphisms in genes encoding miRNAs have been found to be associated with psychiatric disorders [55]. For example, a recent GWAS of schizophrenia identified five new schizophrenia-associated loci, one of which, rs1625579, was located within an intron of a putative primary transcript for the miRNA miR-137 [56]. If these genetic polymorphisms are indeed genuinely associated with psychiatric disorders, they would qualify as secondary epimutations (heritable defects in gene expression due to changes in the DNA sequence of a gene that affect the epigenetic machinery of a cell) [57,58]. A database called MirSNP makes publicly available a collection of polymorphisms that alter miRNA target sites and identifies miRNA-related single nucleotide polymorphisms in GWAS single nucleotide polymorphisms and eQTLs. However, in this context, it must be mentioned that, to date, genetic mapping studies have not definitively identified any locus predisposing to schizophrenia, bipolar disorder, and major depressive disorder [59–61].

Conclusions and future directions

Non-coding RNAs are one of the components of epigenetic mechanisms of regulation of gene expression, and they play a major role in the development and functioning of the brain. They are also involved in the pathogenesis of psychiatric disorders. At present, most research on the role of ncRNAs in psychiatric disorders has focused on miRNAs. This area of research is in its infancy and at present no definite conclusions can be drawn about the precise roles of ncRNAs in the pathogenesis

of psychiatric disorders. Much more work needs to be done on the roles of the different types of ncRNAs in the pathogenesis of psychiatric disorders, by correlating molecular pathophysiology in postmortem brain and peripheral tissues with clinical status of patients with these disorders. Future work on the role of ncRNAs in psychiatric disorders will have to determine the precise ncRNAs dysregulated in different psychiatric disorders and also how the dysregulated ncRNAs interact with other epigenetic mechanisms such as DNA methylation and histone modifications, leading to changes in gene expression and abnormal profiles of proteins in the brain in psychiatric patients.

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Glossary

microRNA A non-coding RNA belonging to the short non-coding RNA category; it is 21–23 nucleotides (nt) in length.

Non-coding RNA RNAs that are transcribed from DNA but are not translated into protein.

CHAPTER

Epigenetics of Bipolar Disorder 13

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KEY CONCEPTS

- The fluctuating course and the evidence that stress can play a triggering role in bipolar disorder suggest the possibility of an epigenetic component to pathogenesis and pathophysiology.
- There was early evidence from parent-of-origin specific inheritance studies and linkage studies that raised the possibility of genomic imprinting in bipolar disorder.
- Molecular data on DNA methylation and histone modifications in bipolar disorder are sparse to date, both at the level of single genes and genome wide.
- The effects of stress and of mood stabilizing medications may shed light on epigenetic variation in bipolar disorder.
- No definitive results have yet been published that clearly demonstrate a role for epigenetic variation in bipolar disorder etiology or pathophysiology.
- Potentially fruitful future directions for study in this area include examining DNA methylation from the same patients in different phases of the illness, from patients before and after successful treatment with medications such as sodium valproate, from patients before and after major stressors, and from animal models of stress-induced mood disorder.

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ABBREVIATIONS

5-HTTLPR Serotonin-transporter-linked polymorphic region
BDNF Brain-derived neurotrophic factor
BP Bipolar disorder
COMT Catechol-*O*-methyltransferase gene
CpG Cytosine–phosphate–guanine
DDC L-DOPA-decarboxylase
DRD4 Dopamine receptor gene, type 4
ECT Electroconvulsive therapy
FKBP5 FK506 binding protein
GAD1 Glutamic acid decarboxylase 1
GR Glucocorticoid receptor
GWAS Genome-wide association study
H3K4me3 Histone H3 lysine 4 trimethylation
HCG9 Human leukocyte antigen complex group 9 gene
HDAC Histone deacetylase
HLA Human leukocyte antigen
HPA Hypothalamic–pituitary–adrenal
HTR2A Serotonin receptor 2A gene
HTR2C Serotonin receptor 2C gene
LOD Logarithm of the odds
PTSD Posttraumatic stress disorder
SNP Single nucleotide polymorphism
SZ Schizophrenia

The epigenetic hypothesis in bipolar disorder

Bipolar disorder (BP) is one of the major psychiatric illnesses, and it is characterized by periods of elevated mood, activity, and cognitive energy that, in their severe form, are called manias and in their milder form are called hypomanias. These typically alternate with periods of low mood, activity, and cognitive energy called depressions. The characteristic "high" periods are characterized by: (1) inflated self-esteem or grandiosity, (2) decreased need for sleep, (3) increased talkativeness or pressure to keep talking, (4) flight of ideas or subjective experience that thoughts are racing, (5) distractibility, (6) increase in goal-directed activity or psychomotor agitation, and (7) excessive involvement in pleasurable activities that have a high potential for painful consequences. People with mania are given the diagnosis of bipolar I disorder, and this illness occurs in 0.5–1.5% of the population worldwide. People with hypomanias are given the diagnosis of bipolar II disorder, while others with features of illness that do not meet full diagnostic criteria are said to have subthreshold bipolar disorder. These conditions have been reported to affect 0.4–1.1% and 1.4–2.4% of the population, respectively [1,2]. The onset of the illness is typically between ages 10 and 30 [3], with the most common period of onset being age 15 to 20 [4]. This delayed onset of BP suggests the potential role of regulation in neural plasticity and a possible effect of hormonal change on brain development. This chronologically dynamic characteristic of the illness suggests the role of dynamic biological processes in the brain. The episodic nature of bipolar disorder is characteristic of the illness. Depressive episodes are typically more frequent and last on average 4–5 months. Manic episodes last about 1–2 months, and hypomanic episodes are shorter and more frequent [5].

Because family, twin, and adoption studies have provided strong evidence for the importance of genetic factors in the etiology of BP, it was among the first illnesses in psychiatry to be targeted for genetic study. But, despite an estimated heritability of 0.75 [6], pinpointing the specific genetic causes of BP has proven to be a formidable challenge. Recent genome-wide association studies (GWASs) have strongly implicated variants in several genes, though, including *CACNA1C* and *ODZ4* [7]. The study of rare variants is ongoing, and that too should inform studies of BP etiology. But, even though genetic information is clearly critical in understanding the development of BP, there are reasons to think this will not tell the whole story. We have mentioned clinical clues that

suggest underlying dynamic biological processes. There is also the observation of a lack of complete concordance for the illness in identical twins [8,9], suggesting that the etiology of BP cannot be explained strictly by static genetic predisposition. The potential role of the environment must be considered. Particularly intriguing is a role for stress and how a stressful environmental might influence the biology of the brain. It has been noted that stressful life events, such as the experience of childhood abuse, can increase the risk of BP and make the prognosis of the illness worse [10]. Among BP patients, those who endorsed a history of child or adolescent physical or sexual abuse, compared with those who did not, had a history of an earlier onset of BP, an increased number of comorbid psychiatric and medical conditions, including drug and alcohol abuse, faster cycling frequencies, and a higher rate of suicide attempts [11]. This observation was replicated by another report showing that compared with those without child adversity, patients who have a childhood abuse history showed an earlier age of onset, more rapid cycling, and more suicide attempts [12]. These consistent data across different populations suggest a relationship between stress and outcomes in BP.

Epigenetic mechanisms

Epigenetic mechanisms play a role in regulation of gene expression in a tissue-specific and age-dependent manner. Among various epigenetic mechanisms, DNA methylation and histone modifications are the ones most commonly studied and described in the literature. DNA methylation is a modification of cytosine on DNA, commonly on CpG sites, and, in general, transcription of DNA is suppressed in correlation with increased methylation of associated CpG sites. Parent-of-origin specific DNA methylation variation has been associated with parent-of-origin specific gene expression in a phenomenon called genomic imprinting. Histone modifications are more complex, as a wide variety of modifications can occur, such as acetylation, methylation, ubiquination, sumoylation, and phosphorylation. Histone methylation itself can be diverse, including monomethylation, dimethylation, and trimethylation. Because a detailed description of these mechanisms is beyond this chapter's scope, for further details please refer to Chapter 2.

Preclinical animal models suggest that stressful environmental factors, such as suboptimal maternal care, can impact the expression of the glucocorticoid receptor gene in rat hippocampus, leading to higher reactivity with elevated cortisol levels in response to restraint stress [13]. Epigenetic mechanisms, including changes in DNA methylation and histone modifications, have been shown to be involved in the regulation of these gene expression changes. These data suggest a potential role for epigenetics in the changes triggered by a stressful environment. Further, experimental administration of exogenous glucocorticoids to mice resulted in a change in DNA methylation levels in the FK506 binding protein gene (*FKBP5*), accompanied by a change in *FKBP5* expression in mouse brain [14]. These data indicate a role for glucocorticoids in the regulation of gene expression through epigenetic modifications.

In humans, there are data from peripheral tissue samples showing an association between epigenetic change and stress. DNA methylation in the glucocorticoid receptor gene (*NR3C1*) was reported to be associated with a history of childhood abuse among depressed patients and borderline personality disorder patients [15]. From postpartum brain samples, it was also reported that *NR3C1* was hypermethylated among suicide completers with a history of childhood abuse compared with those without such adversity [16]. There was also a report that neonatal methylation of *NR3C1* was associated with prenatal exposure to maternal depression and that the cortisol stress response was associated

with methylation levels [17]. Also, some human studies using peripheral blood samples have shown epigenetic changes in several genes associated with immunity and inflammation among people with posttraumatic stress disorder (PTSD) [18,19]. These results support the idea that epigenetic change in the regulation of gene expression is crucial for stress reactivity and for mood and anxiety disorders. Taken together, the relationship between stress and BP, as well as the relationship between stress and epigenetics, supports a potential role for epigenetic modifications in the etiopathogenesis of BP.

Another intriguing clue suggesting the potential role of an epigenetic mechanism in the pathophysiology of BP is that valproic acid, one of the mood stabilizers widely used for the treatment of BP, has a major impact on the epigenome. It is a histone deacetylase (HDAC) inhibitor, thus it increases histone acetylation, thereby broadly promoting gene expression [20]. Valproic acid also leads to demethylation of DNA [21,22]. Thus, it is possible that the treatment effect of valproic acid for BP may be attributable to this epigenetic function of the medication, although there is no direct evidence for this at present.

In summary, several characteristics of BP suggest a role for epigenetics in etiopathogenesis: (1) the later onset of illness in the teens or early 20s; (2) the episodic and recurrent nature of symptoms over time; (3) the impact of stress on severity and prognosis of illness course over time, coupled with the impact of stress on epigenetic marks; (4) the lack of complete concordance for BP in monozygotic twins; and (5) the fact that a mood stabilizer that is effective for the treatment of BP, valproic acid, has a pharmacological function as an HDAC inhibitor. See Figure 13.1.

Direct examination of epigenetic impact from the study of bipolar disorder

Early evidence suggesting a role for epigenetics in BP came from the study of parent-of-origin specific linkage patterns in BP pedigrees. Studies of chromosome 18 using a Johns Hopkins sample found that linkage to chromosome 18q21–22 came predominantly from paternally inherited alleles [23]. A subsequent study, focusing on a phenotypically defined subgroup of families, obtained a strong LOD score for paternally transmitted alleles and near zero for maternally transmitted alleles [24]. This result suggested the possibility that an imprinted gene in the region could account for the linkage in the 18q22 region. Two BP genome scans have examined parent-of-origin specific linkage. One study identified two regions (2p21–24 and 2q31–32) that showed suggestive evidence for linkage when only maternal transmission was examined, and two regions (14q32 and 16q21–23) that showed suggestive linkage when only paternal regions were examined. In the Johns Hopkins sample, two regions (1q42 and 13q12) showed linkage with maternally transmitted alleles, and one region (18q21–22) showed linkage with paternally transmitted alleles. The 13q12 region contains *5HT2A*, reported to be imprinted in fibroblasts and in brain tissue from some subjects but not others. One association study of *5HT2A* examined whether a parent-of-origin specific association could be detected in BP subjects; results were negative.

Several association studies have reported parent-of-origin specific evidence for association with BP. One study examined *DDC*, which codes for L-DOPA-decarboxylase, and another assessed *DRD4*, the dopamine receptor gene, type 4. Both genes are biologically plausible candidates, and both lie adjacent to known imprinted genes. A study assessing imprinting of *DRD4*, however, found evidence for normal, non-imprinted expression of the gene. A more complex scenario of brain region-specific,

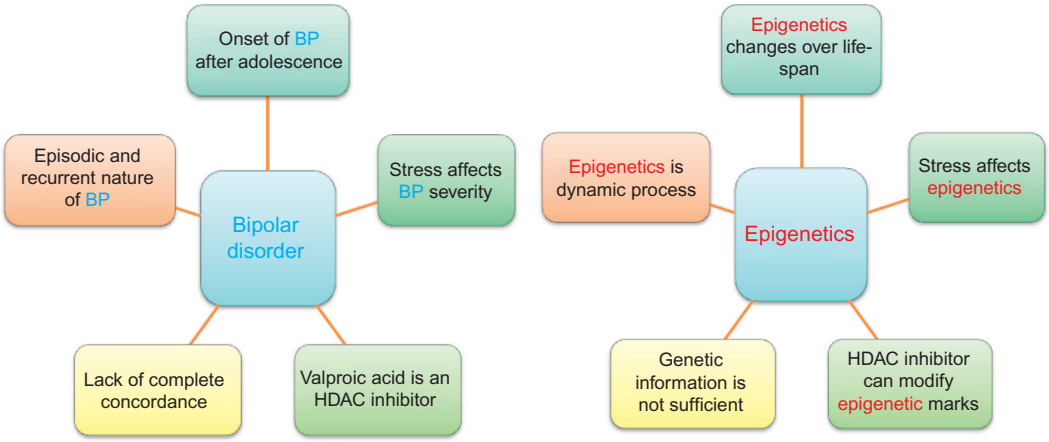
Onset of BP after adolescence
Episodic and recurrent nature of BP
Bipolar disorder
Stress affects BP severity
Lack of complete concordance
Valproic acid is an HDAC inhibitor
Epigenetics changes over life-span
Epigenetics is dynamic process
Epigenetics
Stress affects epigenetics
Genetic information is not sufficient
HDAC inhibitor can modify epigenetic marks

FIGURE 13.1

Several conceptual reasons to suspect that epigenetics might play a role in the etiopathogenesis of bipolar disorder are shown. On the left are aspects of bipolar disorder course of illness, genetics, and treatment, and on the right are aspects of epigenetics that could potentially be relevant for the corresponding factors in bipolar disorder. Correspondence is indicated by matching positions.

developmental stage-specific, or alternative transcript-specific imprinting cannot be ruled out. Loci on chromosome 18p11 have been associated with BP at the suggestive level when parent-of-origin association analyses were employed in a family-based sample [25]. Other BP investigators have observed potentially imprinted genes on 18p11 [26]. Further investigation is needed to determine whether genomic imprinting indeed plays a role in BP etiology.

The direct study of epigenetic variation at the molecular level is challenging in BP, as it is for all psychiatric disorders, because the epigenetic status of brain tissues is not necessarily the same as that of other peripheral tissues. Although the theoretical ideal tissue to study is the living brain of patients, it is generally not possible to do so for obvious reasons. One alternative approach is to study postmortem brain tissues from patients. Ladd-Acosta et al. [27] showed that, even then, DNA methylation differences could be detected between brain regions within the same individuals; however, the majority of CpGs did not vary, suggesting that most epigenetic marks are consistent across the brain. Another approach is to study peripheral tissues. Again, although tissue-specific differences exist for some epigenetic marks, for many others there is consistency across tissues. For example, one study showed that DNA methylation levels of the catechol-*O*-methyltransferase (*COMT*) gene in prefrontal cortex and in peripheral blood mononuclear cells of rats were correlated [28]. Another study investigated genome-wide DNA methylation status across various tissues, including different regions of brain as well as whole blood. The resulting data suggested that, although methylation patterns are often tissue specific, some of the differences in DNA methylation between individuals are correlated across brain and blood [29].

Additional challenges include the influence of exposure to substances and to medications, as well as the influence of age. It is well known that patients with BP have a high rate of comorbid substance use disorder. Thus, many of them are exposed to substances such as alcohol, nicotine, and illicit drugs.

It is not well understand how those substances might influence epigenetic status in the brain and in other tissues. Also, because of their BP, many of these patients are treated with a wide range of psychotropic medications, such as mood stabilizers, antidepressants, and antipsychotics. Exposure to medications such as valproic acid would likely modify epigenetic marks. Epigenetic status is known to be dependent on developmental stage and on age. One study using postmortem brain across the lifespan, ranging from fetal periods to old age, showed that DNA methylation of the frontal cortex changes at the fastest rate during the prenatal period, followed by a slower rate after birth, but continues to change through the lifespan, albeit at a much slower rate [30]. These data suggest that DNA methylation variation is a lifelong dynamic process. In spite of these challenges, epigenetic approaches to the molecular investigation of the etiopathogenesis of BP are being implemented.

Single-gene DNA methylation studies and bipolar disorder

A candidate gene approach was employed for most of the earlier studies that investigated an association between DNA methylation and BP. Results are summarized in Table 13.1. Typically, samples from schizophrenia (SZ) and BP subjects have been compared with those from healthy controls. Hypomethylation of the *MB-COMT* gene was initially reported in the frontal lobe from postmortem brain [31], followed by similar results obtained using saliva [32] with SZ and BP. The same group also investigated a methylation change in the serotonin receptor 2A gene (*HTR2A*) from postmortem brain tissue [33] and from saliva [34] with SZ and BP. However, the results did not show straightforward associations. For example, one locus within the gene showed hypermethylation, while another showed hypomethylation. Also, the results were not consistent between brain and saliva.

Another serotonin receptor gene, *HTR1A*, was studied by a group from Switzerland. The study used peripheral blood and showed increased DNA methylation levels among SZ and BP samples [35]. A group from Italy investigated the brain-derived neurotrophic factor gene (*BDNF*) using DNA from peripheral blood mononuclear cells. They found that BP II subjects showed the highest methylation compared with both BP I subjects and controls. Interestingly, patients treated with mood stabilizers (both lithium and valproic acid) showed lower DNA methylation than those without such treatment [36].

Kaminsky et al. [37] studied the human leukocyte antigen (HLA) complex group 9 gene (*HCG9*) in BP. They analyzed brain, blood, and sperm and reported similar DNA methylation changes (hypomethylation) across tissues associated with BP subjects when compared with controls. A group from Japan led by Kato [38] studied lymphoblastoid cell lines derived from monozygotic twins discordant for BP and found that the serotonin transporter gene was hypermethylated in BP twins. Of interest, they reported that people who were homozygous for the short allele of the serotonin transporter promoter region polymorphism (5-HTTLPR) showed higher methylation levels and lower expression levels than those with the heterozygous genotype. This is consistent with literature showing that genotype mediates some epigenetic variation.

One of the important aspects of epigenetics is its role in the context of gene–environment interaction. Among many environments, the most studied and the most relevant for psychiatric conditions including BP is childhood maltreatment. A recent report showed that the higher the number of traumatic events, the higher was the methylation level of the glucocorticoid receptor gene in peripheral blood leukocytes for individuals with BP [39].

Table 13.1 Previous BP Studies on DNA Methylation in Single Genes

Study	Gene Studied	Region (Tissue)	Methylation	Patient Group
Abdolmaleky et al. [31]	*MB-COMT*	Brain (SMRI)	↓ (frontal cortex)	Compared with control
Nohesara et al. [32]	*MB-COMT*	Saliva	↓	Compared with control
Abdolmaleky et al. [33]	*HTR2A*	Brain (SMRI)	↓↑ (frontal cortex)	Compared with control
Ghadirivasfi et al. [34]	*HTR2A*	Saliva	No differences reported	Compared with control
Carrard et al. [35]	*HTR1A*	Blood	↑	Compared with control
D'Addario et al. [36]	*BDNF*	Blood mononuclear cells	↑	BP II Compared with BP I and control
Ferreira et al. [40]	*BDNF*	Brain (McL)	↑	Compared with control
Ferreira et al. [40]	*COX-2*	Brain (McL)	↓	Compared with control
Ferreira et al. [40]	*Drebrin*	Brain (McL)	↑	Compared with control
Kaminsky et al. [37]	*HCG9 (HLA complex group 9)*	Brain (SMRI and McL), WBCs, and sperma	↓ (frontal cortex)	Compared with control
Sugawara et al. [38]	*SLC6A4*	Lymphoblastoid cell lines	↑	Compared with control
Perroud et al. [39]	*NR3C1*	Blood leukocytes	↑	BP with/without childhood abuse history

Abbreviations: McL, McLean 66 Cohort Collection of the Harvard Brain Tissue Resource Center; SMRI, Stanley Medical Research Institute; WBCs, white blood cells.

The challenge associated with these epigenetic studies targeting single genes is that until very recently there have not been definitive genes associated with increased risk for BP to study. However, now some are emerging from GWASs, such as *CACNA1C* [40] and *ODG4* [7]. Investigation of epigenetic modifications at these candidate genes discovered through GWASs could be of great interest. Although little has been done to date on these genes, one report has investigated differences in DNA methylation of *CACNA1C* between neurons and non-neurons from mouse brain. The report showed that one CpG island and shore around exon 1 had brain cell-type-specific methylation differences; [41] however, no studies of these genes have yet been reported for BP itself.

Genome-wide methylation studies and bipolar disorder

One approach to measuring DNA methylation levels across the genome is to obtain global DNA methylation data. A few such studies have been done. One study measured global DNA methylation from frontal cortex of postmortem brain from BP patients as compared with matched controls.

The data showed increased global DNA methylation in BP [42]. Interestingly, the investigators compared brain samples from Alzheimer's disease as well, and they showed similar epigenetic profiles as with BP. Another study measured global leukocyte DNA methylation from euthymic BP patients. The result showed no alteration of global DNA methylation level in that population when compared with matched healthy controls [43]. This global approach provides no gene-specific or sequence-specific information. To obtain a fine-grained picture, a locus-specific genome-wide approach is required.

There have been two locus-specific genome-wide DNA methylation studies in BP [44,45]. In the first study of this kind, Mill et al. [45] investigated DNA methylation changes in the frontal cortex and germline from SZ and BP patients using CpG island microarrays. They reported association of DNA methylation differences at numerous loci with BP in sex-specific analyses. Some of those loci were related to glutamatergic and GABAergic neurotransmission and brain development. The data also suggested methylation variation related to stress and to lifetime exposure to antipsychotics. Another study using a genome-wide approach investigated epigenetic profiles of whole blood among discordant identical twins with BP as well as SZ. Interestingly, some CpG sites showed a high level of methylation difference for both BP discordant twins and SZ discordant twins, but in opposite directions. For example, a promoter region of *ZNF659* had a CpG site that showed hypomethylation in the BP twins but hypermethylation in the SZ twins [44]. These data suggest the possibility that specific disorders may have specific epigenetic patterns, although replication is needed to confirm these associations.

Histone modifications studies and bipolar disorder

While there are a great many histone modifications known to exist, only a few have been the focus of extensive study. These are acetylation and several types of methylation. When histone acetylation occurs, DNA, bound to histone, is more "relaxed," and transcription factors can access transcription start sites more easily, allowing more efficient transcription of genes. There is an equilibrium relationship between acetylated and de-acetylated histone, and enzymes, such as HDACs, play a role in the balance between the two phases. One study investigated global histone H3 acetylation as well as phosphorylation in postmortem frontal cortex among BP samples, which showed heightened histone modifications of both forms in BP when compared with matched controls [42]. As mentioned earlier, this study compared Alzheimer's disease samples as well, and the pattern of histone modifications detected was similar to those of BP samples with regard to phosphorylation, although increased histone acetylation was unique to BP samples. Another study investigated histone acetylation status at specific genomic sites, mainly chosen from candidate genes associated with SZ [46]. These investigators compared postmortem human brain samples from BP, as well as from SZ, with controls. Their data showed that some of the candidate genes, such as glutamic acid decarboxylase 1 (*GAD1*) and serotonin receptor 2C (*HTR2C*), were more highly expressed when levels of histone acetylation were higher, but this held across all samples; no differences were seen between BP cases and controls.

Histone methylation includes several variations (e.g., monomethylation, dimethylation, and trimethylation), and these can occur at a number of lysine positions, such as histone H3 lysine 4 and histone H3 lysine 27. Only one published study has addressed histone methylation changes in BP. Investigators studying postmortem brain showed a significant association of increased histone H3 lysine 4 trimethylation (H3K4me3) at the synapsin gene (*SYN2*) with BP and this correlated with increased gene expression [47]. See Table 13.2.

Table 13.2 Previous BP Studies on Histone Modification

Study	Gene and Rank Studied	Tissue Source	Histone Modification	Patient Group
Ferreira et al. [40]	Global histone acetylation and phosphorylation	Brain (McL)	↑ (frontal cortex)	Compared to control
Tang et al. [46]	*GAD1* *H3K9K14ac*	Brain (McL)	No difference reported (frontal cortex)	Compared to control
Tang et al. [46]	*HTR2C* *H3K9K14ac*	Brain (McL)	No difference reported (frontal cortex)	Compared to control
Cruceanu et al. [47]	*Synapsin (SYN2)* *H3K4me3*	Brain (QSB)	↑ (frontal cortex)	Compared to control and major depressive disorder

Abbreviations: H3K4me3, histone H3 lysine 4 trimethylation; H3K9K14ac, histone H3 lysine 9 and 14 acetylation; McL, McLean 66 Cohort Collection of the Harvard Brain Tissue Resource Center; QSB, Quebec Suicide Brain Bank.

Epigenetics of stress as an entry point

As noted, stress may play an etiologic and/or pathophysiologic role in BP. Thus the impact of stress on epigenetic marks may be relevant to these potential roles. We and others have examined the stress-related gene *FKBP5* in this context. FKBP5 is a co-chaperone protein that forms a complex with the glucocorticoid receptor (GR) and regulates its activity. When it is attached to inactive GR, it reduces GR affinity for glucocorticoids and also decreases overall GR signaling by reducing GR presence in the cytoplasm through nuclear translocation [48]. Thus, a high level of FKBP5 can lead to low sensitivity of the hypothalamic–pituitary–adrenal (HPA) axis to glucocorticoids and thereby make this negative feedback system less effective. Of interest, *FKBP5* has functional polymorphisms (such as rs1360780) that have been widely studied in relation to depression, PTSD, and stress reactivity. Binder et al. [49] showed that depressed subjects with the risk allele polymorphism for rs1360780 who express higher *FKBP5* experience more depressive episodes than those who have other genotypes. In a separate study, they also showed that the risk of PTSD increased in a "dose-dependent" manner along with the number of the risk alleles of rs1360780 among patients exposed to childhood adversity in a significantly traumatized community sample [48]. Willour et al. [50] reported that single nucleotide polymorphisms (SNPs) in *FKBP5* are associated with risk for BP.

In an animal model, Lee et al. [14] showed that exogenous steroid administration to mice can modify DNA methylation in *FKBP5* within brain as well as peripheral blood. Binder's group showed that stress can elevate *FKBP5* expression by altering the DNA methylation pattern at the *FKBP5* locus. Childhood adversity has been observed to decrease DNA methylation at a regulatory element in intron 2 and 7 of *FKBP5*, increasing the gene's expression in response to GR signaling and thus dampening the negative feedback mechanism of the HPA axis [51].

In addition to *FKBP5*, stress can also disrupt suppression of HPA axis activity by altering DNA methylation at the promoter of the GR gene *NR3C1*. Initially, Weaver et al. [13] showed that suboptimal maternal care of rat offspring altered DNA methylation at a GR gene promoter in the hippocampus that led to heightened HPA axis reactivity. Following on the animal study, a human postmortem

study showed similar data from victims of childhood abuse who committed suicide. Compared with suicide completers without a history of childhood abuse, those with a childhood abuse history showed higher DNA methylation at the *NR3C1* promoter and decreased GR expression [16]. Data from peripheral blood show a similar change of DNA methylation in *NR3C1* associated with a history of childhood abuse among BP patients [39].

Together, these data from both human and animal research underscore the important role of epigenetic modification in the stress response. Given the role of stress in the onset, illness course, and prognosis of BP [10–12], investigating a potential role for epigenetic mechanisms in BP from the perspective of stress reactivity is of interest.

Epigenetics of neuroplasticity

Another physiological process that may mediate between environmental stressors and behavioral change is epigenetic change within neuroplasticity factors. Neuroplasticity provides cellular adaptation to environmental signals such as stressful stimuli. A gene recognized as a major player in neuroplasticity is the brain-derived neurotrophic factor gene (*BDNF*) [52]. *BDNF* is a neurotrophin that is highly expressed in the central nervous system and mediates a number of cellular adaptations to facilitate brain adaptation to environmental signals. Because BDNF regulates neuroplasticity processes, changes to its expression by stress-induced DNA methylation patterns can have an impact on behavior. In a study where rat infants were exposed to early-life stress in the form of maternal maltreatment, stressed rats exhibited anxiety behavior and showed reduced *BDNF* mRNA expression in the prefrontal cortex corresponding to increased DNA methylation at promoter IV and XI compared with control rats [53]. Similarly, in a separate study, rats exposed to chronic stress in the form of chronic inescapable cat exposure showed a reduced level of *BDNF* exon IV transcript in the dorsal dendate gyrus and dorsal CA1. DNA methylation changes were, however, absent in ventral dentate gyrus and ventral CA1 [54]. This study highlights the tissue specificity of stress-induced DNA methylation changes, suggesting that tissue heterogeneity may potentially mask this difference from being observed. In line with this idea, a separate study that investigated the effect of chronic social defeat stress in whole hippocampus of mice did not observe any DNA methylation differences between stressed and control mice [55].

In addition to animal studies, clinical studies have also linked the effect of *BDNF* DNA methylation patterns to psychiatric phenotypes. For example, bipolar II patients had increased DNA methylation in *BDNF* promoter I in peripheral blood [36]. In addition, a recent genome-wide DNA methylation study using premortem blood and postmortem human brain tissues observed similar inter-individual differences in DNA methylation pattern between the two tissues [29]. These studies suggest that changes to DNA methylation pattern at the *BDNF* locus detected in blood may reflect its changes in the brain of BP patients. These data suggest that altered DNA methylation in *BDNF* can negatively impact behavior and thus might be relevant for epigenetic investigation of BP.

Future directions

In this last section, we summarize important strategic approaches in the epigenetic investigation of psychiatric conditions including BP. First, the integration of genetic information and epigenetic

information is needed [56]. Genotype variations such as SNPs, and epigenetic marks, such as DNA methylation and histone modifications, are not exclusive of each other, but rather interacting factors in the control of gene expression and subsequent cell functions leading to various phenotypes. Thus, integration of genetic and epigenetic information could be vital to understanding BP etiopathogenesis. As shown with the examples of *FKBP5* and *BDNF*, it is suggested that those candidate genes identified through genetic association studies, either through traditional association studies that look into main effects or through approaches considering gene–environment interaction, can also be good candidate genes for epigenetic study. Thus, combining data from GWASs and epigenome-wide association studies will be important for going forward.

One useful strategic approach to eliminating the impact of genetic variability on epigenetic marks is to compare the same individuals before and after exposure to particular environments. These exposures could be: (1) treatments such as medication, electroconvulsive therapy (ECT), or psychotherapy; (2) substances such as alcohol and illicit drugs; and (3) stressful life events, such as childhood abuse, rape, or combat exposure. The resulting data could shed light on etiopathogenesis or provide biomarkers that might correlate with course of illness, treatment response, or the development of side effects.

One interesting approach to simulating a stressful environment would be accomplished by administration of exogenous glucocorticoid. Natural experiments of this type can be found in medical/surgical settings, such as in neurosurgical patients before brain surgery for tumor resection or treatment refractory epilepsy, or patients with acute symptoms of ulcerative colitis who require a large intravenous (IV) bolus dose of glucocorticoid. The role of steroids in mood change is intriguing, because it is often encountered in a psychiatric consultation setting that a patient administered IV glucocorticoids develops manic symptoms and requires psychiatric evaluation. Asthma patients or rheumatoid arthritis patients taking regular oral glucocorticoids present another natural experiment. Comparison of these two different medical settings would be analogous to comparing the acute effects of significant trauma to the long-term effects of chronic mild-to-moderate stress. Such studies would have to take into account how these exogenous glucocorticoids compare with physiological cortisol levels under stressful environments.

Another approach that could help shed light on BP etiopathogenesis would be to examine the epigenetic status measured in a manic episode, in a depressed phase, and in euthymic states for the same individuals. Differences seen in particular genes might point to key loci responsible for mood variation.

Conclusions

Overall, no definitive data have yet emerged from the epigenetic study of BP. For example, no replication data for single gene epigenetic signals are available. The study of candidate genes that arise from positive signals identified through GWASs is a promising route. Epigenome-wide efforts need to be continued to better understand the landscape of relevant epigenetic variation across the genome. Integration of genetic variation and epigenetic modifications, including both DNA methylation and histone modifications, will be important. Epigenetic investigation of stress models as key elements of environmental influence is promising. Further, therapeutic models that compare epigenetic marks before and after interventions are also of great interest for the epigenetic study of BP.

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Glossary

CpG island Region with a high proportion of cystosine–guanine dinucleotides.
Epigenome The collection of all DNA methylation variations and/or all histone variants across the genome.
Glucocorticoid A steroid hormone that is central to the stress response.
Hypothalamic–pituitary–adrenal axis A set of interactions among the hypothalamic region of the brain with the pituitary and adrenal glands that is mediated by hormones released in response to stress.
Posttraumatic stress disorder A psychiatric condition that arises in response to experiences such as combat or assault and is characterized by hyperarousal, flashbacks, and avoidance.
Sodium valproate/valproic acid A medication used to treat epilepsy and bipolar disorder that is known to have inhibitory effects on histone deacetylase.

CHAPTER

Epigenetics in Major Depressive Disorder

14

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KEY CONCEPTS

- Epigenetics is thought to play a key role in the pathogenesis of major depressive disorder.
- Epigenetic mechanisms of gene expression are thought to mediate the role of environment factors in the pathogenesis of this disorder.

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ABBREVIATIONS

5-HT Serotonin
ACTH Adrenocorticotrophic hormone
BDI Beck Depression Index
BDNF Brain-derived neurotrophic factor
BOLD Blood oxygenation level dependent
CBP CREB-binding protein
CHARM Comprehensive high-throughput array for relative methylation
CREB cAMP response element-binding protein
CRF Corticotropin-releasing hormone
DZ Dizygotic
E2 Estradiol
EPDS Edinburgh Postnatal Depression Scale
ER Estrogen receptor
FACS Fluorescent-activated cell sorting
fMRI Functional magnetic resonance imaging
GR Glucocorticoid receptor
GWASs Genome-wide association studies
H3K27 Histone H3 lysine 27
H3K9 Histone H3 lysine 9
HATs Histone acetyltransferases
HDACs Histone deacetylases
HMTs Histone methyltransferases
HPA axis Hypothalamic–pituitary–adrenal axis
IL-1α Interleukin 1α
LASS2 Longevity assurance homolog 2 gene
LGA Large for the gestational age
LLD Late-life depression
LTP Long-term potentiation
MAOIs Monoamine oxidase inhibitors
MDD Major depressive disorder
MDE Major depressive episode
MeDIP Methylated DNA immunoprecipitation
MOR μ-Opioid receptor
MZ Monozygotic
NGFI-A Nerve growth factor-inducible protein A
NNNS NICU Network Neurobehavioral Scales
OVX Ovariectomized
P4 Progesterone
PCTK1 Cyclin-dependent kinase 16 gene
PET Positron emission tomography
PFC Prefrontal cortex
PPD Postpartum depression
PTSD Posttraumatic stress disorder
SGA Small for the gestational age
SSRIs Selective serotonin reuptake inhibitors
VTA Ventral tegmental area

Introduction

Major depressive disorder (MDD) is the most prevalent psychiatric illness in the United States and is estimated by the World Health Organization to be the largest cause of disability worldwide, with over 350 million people affected [1]. MDD, like most psychiatric diseases, is a complex non-Mendelian disease and is believed to result from a combination of factors including underlying genetic risk and exposure to environmental factors that lead to development of depressive symptoms through their various interactions. Although the precise substrates of these susceptibilities of "nature" and "nurture" have not fully been identified, it is the interplay between these two seemingly unrelated forces of development that most likely underlie the disorder. At the heart of this interplay is the growing field of epigenetics. Epigenetics has always been closely linked to the environment's influence on development but it is only in the last few decades that the scientific community has come to understand the nature of this interaction. In one sense, epigenetics may be considered to be a mere molecular record of environmental influence or simply a molecular surrogate for the term environment, such that gene X environmental interactions may be thought of as gene X epigenetic interactions; however, such a substitution may be out of place. An apt analogy may be to consider the malleable epigenetic landscape like a dirt road, and the surrounding environment made up of cars, foot traffic, and weather ultimately leave their mark. Any altered condition of the epigenetic road of life resultant from external pressures will

feed back to affect the experience of the travelers. Furthermore, the type of road an individual travels through life may be inherited to some extent from their parents. Whereas genetic predispositions may determine some aspects of the building material, the weather conditions during road construction influenced by the intrauterine environment may result in a new road for a child with potholes already beginning to form. This chapter will highlight how both genes and environment may influence the epigenetic landscape and how this in turn may lead to MDD. Specifics regarding the effects of intrauterine diet and stress and early postnatal environment on epigenetic reprogramming will be highlighted, and their subsequent consequences will be explored in the context of MDD-specific endophenotypes of neural connectivity related to altered mood.

What is major depression?

The *Diagnostic and Statistical Manual of Mental Disorders IV* (DSM-IV) classifies MDD as a mood disorder, which relates to disorders that exhibit extreme ranges in mood. In MDD, mood extremes can include a loss of energy, sadness, anhedonia (an inability to experience pleasure), thoughts of suicide, and a general impairment of sleep, concentration, attentiveness, or decision making. MDD diagnosis requires a major depressive episode (MDE), defined as a discrete and pervasive period of these symptoms; however, heterogeneity exists in the context in which MDEs occur, and the specific symptoms inherent in each may differ, suggesting that considerable heterogeneity may exist in the underlying architecture of the DSM-IV classification of MDD. For example, up until puberty the rates of MDD are relatively equivalent between males and females, after which rates for females double or triple [2–4]. Postpartum depression (PPD) is a specific class of female-specific mood disorder where MDE occurs within 4 weeks of giving birth. The onset of MDD after the age of 50 to 60 has been termed late-life depression (LLD). These separate classes of depression may share common genetic and environmental foundations to confer risk, but they may also have distinct etiologies resulting in their different presentation. Where applicable throughout the chapter, evidence to this effect will be highlighted.

Brain imaging endophenotypes in MDD

Through neuroimaging studies in the past 15 years, a sizable catalog of brain activity endophenotypes in MDD has been accumulated. The results of various meta-analyses indicate that activity differences have been observed using functional magnetic resonance imaging (fMRI) with MDD in a number of regions of the prefrontal limbic network thought to modulate cognitive function and emotional processing. Specifically, these relate to an increased activity of the amygdala, subgenual anterior cingulate cortex, and ventral medial prefrontal cortex with a concomitant decrease in activity of the dorsolateral and dorsal medial prefrontal cortex, as well as the dorsal and rostral anterior cingulate cortex [5]. The hypothesized consequence of these observations is an MDD-specific failure of cognition associated with dorsal inhibition on medial structure-mediated emotional processing [6]. Specific models suggest that alterations of these processes are modulated by either automatic or voluntary attempts to control stimuli [7]. In a systemic review of MDD imaging data, Rive et al. [7] analyzed imaging studies directed toward assessing either automatic or voluntary processing tasks as initially

introduced by Phillips et al. [8]. Medial prefrontal regions responsible for automatic processing of emotional stimuli sequester additional resources from the lateral prefrontal structures in MDD, while voluntary emotional processing tasks demonstrate a decreased activity of lateral prefrontal structures and marked task deficits in MDD [7]. Another area exhibiting activity-related differences in MDD is the ventral tegmental area (VTA) [7,9], a major source of dopaminergic neurons in the mesolimbic pathway that mediate response to reward and are thought to be related to the manifestation of anhedonic depressive symptoms. However, the mesolimbic reward circuitry may not be limited to mediating anhedonia, as diffusion tensor imaging in MDD patients identified fractional anisotropy increases in the right VTA that were associated with increased trait anxiety scores but not anhedonia difference [10]. Recent advanced intervention approaches in animal models such as optogenetic analyses are confirming the importance of this region to a broad range of depressive symptoms and behaviors [11].

Two genetically driven hypotheses of depression

On the macro level, there are two main hypotheses that have been put forward to explain MDD symptomology as well as imaging-based endophenotypes—namely, the monoamine hypothesis and the neuroplasticity hypothesis [12]. The monoamine hypothesis states that depression results from a decrease in monoamine levels in depressed patients and originated based primarily on the observation that antidepressants that increased levels of serotonin and norepinephrine had positive effects on mood [12,13]. A key brain structure in these models is the amygdala, where fMRI-based amygdala responses to negative words or stimuli lasted longer in MDD patients over controls and correlated with levels of blood oxygenation level dependent (BOLD) activity [14]. These effects are demonstrated to be abrogated by the administration of antidepressants [14]. A major class of antidepressants are the selective serotonin reuptake inhibitors (SSRIs), which function to increase synaptic serotonin (5-HT) levels through inhibition of 5-HT transporter-based clearance. An insertion/deletion genetic polymorphism of the 5-HT transporter (5-HTT) gene (*SLC6A4*) has been shown to alter 5-HT transporter function and is associated with levels of anxiety and resilience to adversity [15]. The deletion variant (often referred to as the "s" variant, standing for "short") is associated with decreased 5-HT transporter-mediated reuptake into presynaptic cells and increased amygdala responsiveness to negative facial cues in healthy subjects, stressed rhesus monkeys, and MDD cases [14]. 5-HT availability as well as gene expression of the *SLC6A4* gene are not uniformly distributed through the brain but exist primarily in MDD neuroimaging endophenotype-associated brain structures, including the hippocampus, amygdala, medial frontal regions, and anterior cingulate cortex [16], adding further plausibility to the monoamine hypothesis of depression.

Antidepressants that function to immediately increase monoamine levels, such as SSRIs or monoamine oxidase inhibitors (MAOIs), present a problem for the monoamine hypothesis of depression—namely, that the mood-improving effects often require weeks to take effect. If low levels of monoamines are of etiological importance to mood in MDD, then correction of these levels should lead to instant amelioration of depressed phenotype. This problem, along with initial MRI-based observations of volumetric decreases in the frontal cortical regions and hippocampus in depressed patients, led to the neuroplasticity hypothesis, whereby alterations in neural connectivity mediated by depression-associated deficits in neurotrophic factors lead to altered function in brain structures important for modulating

mood and response to rewards [17]. A bulk of the work supporting this hypothesis stems from studies investigating MDD deficits in the brain-derived neurotrophic factor (BDNF) [12]. Levels of BDNF are reduced in postmortem hippocampal samples from MDD patients [18], whereas stress-induced BDNF decreases in animal models can be corrected through administration of antidepressants [17,19]. BDNF plays important roles in long-term potentiation (LTP) through presynaptic modulation of vesicle docking and postsynaptic actions modulated by the Trk-B receptor [20]. It is critical for the development, maintenance, and survival of GABAergic, dopaminergic, cholinergic, and serotinergic neurons [16]. A genetic polymorphism at rs6265 causes a functional Val66Met mutation. The Met version of the protein results in failed intracellular trafficking of BDNF and significantly decreased BOLD response in the medial temporal lobe during episodic memory tasks [20]. There is some indication that the Val66Met polymorphism may mediate anterior cingulate cortex volume [21] as well as extinction learning dependent on the amygdala [22]. Many fMRI studies, however, report no effect of the Val66Met polymorphism on amygdala activation [16]. Although there is compelling evidence to suggest a link between BDNF variation and depression-related phenotypes, factors such as the brain region and antidepressant-specific effects of BDNF call into question BDNF as a unifying risk variant for MDD [12]. Whether alterations in neurotrophic factors or monoamine systems represent etiological causes of depression, or intermediate molecular phenotypes contributing to the disorder, is not known at this point. If the former is the case, it would seem that genetic variation in these systems should account for a large degree of disease variance. As will be outlined below, this does not appear to be the case, leaving the door open for other factors to be considered.

Nature and nurture in MDD

The etiology of MDD is thought to result from the combined influence of genetic and environmental factors as, like most traits, MDD exhibits some degree of heritability. Heritability may be derived from the comparison of monozygotic (MZ) genetically identical with dizygotic (DZ) non-identical twins, which is known as the classical twin design. DZ twins share 50% of their segregating polymorphisms, on average, and as such a comparison of MZ with DZ twin concordance rates for a given trait allows for an estimation of the proportion of a phenotype that is inherited due to genetic factors. Most classical twin studies of MDD determined a heritability of around 30% [23–28]. A recent application of common genetic variation at single nucleotide polymorphisms estimated the heritability for MDD to be lower at 23% [29]. These specific types of "SNP heritability" take advantage of genome-wide genetic sampling techniques in larger samples and speak only of the heritability accounted for by the measured genetic polymorphisms. Conversely, twin studies will identify the presence of all heritable factors independent of their source. The lower estimate, when accounting for a majority of common genetic variation, suggests the possibility for additional sources of transgenerational transmission of phenotype. Interestingly, proband-wise MZ concordance for major depression differs by gender, with 31% for male and 48% for female MZ twins [30], suggesting an increased heritability of depression risk in the female population. Cumulatively, these data suggest that the inheritance of genetic risk factors represents an underlying risk for MDD development. What is not attributed to genetics in heritability studies falls into the category of non-shared environmental influence, and in the case of MDD this portion represents a relatively large (~70%) piece of the pie.

GWAS and the missing heritability

Despite evidence of promising associations of genetic risk factors such as the Val66Met BDNF polymorphism and the *SLC6A4* promoter polymorphism with depression endophenotypes, the confidence in a genetic etiology to depression involving these factors remains small. Genetic analysis of the Val66Met BDNF mutation discussed above is not associated with MDD risk [31,32], and a myriad of studies on the effects of the *SLC6A4* promoter polymorphism have met with conflicting results. Genetic factors such as these may require complex interactions with other underlying genetic risk as well as the environment to induce the depressive phenotype. In the case of *SLC6A4*, initial reports suggested it acted in a gene × environmental manner, interacting with stressful life events to mediate risk to depression [33]; however, numerous studies over the past decade have called this finding into question [34]. It was hoped that with the application of genome-wide association studies (GWASs) to psychiatric phenotypes the underlying genetic determinants of psychiatric phenotype heritability, including MDD, would be uncovered. Unfortunately, the lack of replicating genetic associations to complex traits over the past ~30 years and underwhelming results from GWASs in psychiatric disease since its inception have led to the increasingly occurring issue of "missing heritability" [35].

Missing heritability occurs when the amount of risk attributed to a given trait by family or twin studies is not accounted for by genome scans for genetic variation for that trait. In fact, it is not the heritability that is missing, but the identification of the underlying factors responsible for it. There is little doubt that complex diseases such as MDD are heritable, as there is a higher risk to MDD in first-degree relatives even if those relatives are adopted, but then why have so few genetic variants been discovered? One possibility is that the genetic technologies used thus far have not had the resolution necessary to identify rare genetic variants not detectable by a GWAS, a prospect that has fueled a recent surge of second-generation sequencing studies in complex disease. The next few years will determine if this approach will yield fruitful advances. An alternative interpretation is that genetic risk is mediated by the combined influence of thousands of genetic variants conferring only a small degree of risk for each variant, but that the combined influence of all genetic variation in concert is responsible for manifestation of the genetic component of disease risk. This concept led to the creation of the polygenic risk score that has been successful in predicting and replicating higher levels of genetic risk across a range of psychiatric traits, such as amygdala volume and activity and its connection to the medial prefrontal cortex [36]. However, in a recent review of the current state of genetic evidence in major depression, Cohen-Woods et al. [37] suggested that the conflicting genetic results in MDD to date may be a consequence of a failure to account for environmental influence. Thus, although genetic research is indicating that the combined influence of hundreds or thousands of risk loci may interact to result in deleterious psychiatric consequences, we must not lose sight of the fact that the genome is under epigenetic control, which in turn may be modified by the environment. As such, the phenotypic manifestation of genetic risk factors may be enhanced or blocked through the interaction of DNA and chromatin modifications.

Environmental influence, epigenetics, and the missing heritability

Epigenetic factors are stably inherited through mitosis; however, numerous environmental events can cause both widespread and focused changes to epigenetic signatures, resulting in altered phenotypes.

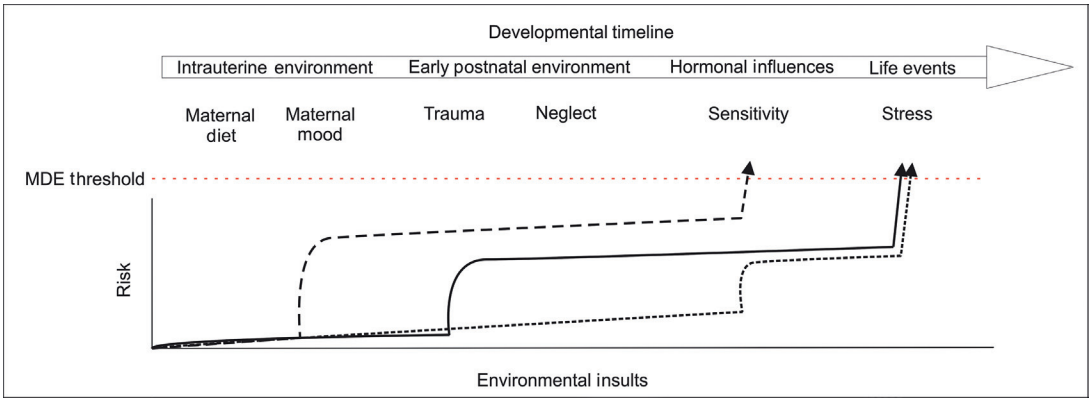

FIGURE 14.1

A hypothetical model of environmentally induced risk to depression. Depression risk (*y*-axis) may be compounded through successive environmental insults (*x*-axis) that serve to increase vulnerability to stressful life events or other major depressive episode (MDE) precipitating events such as hormonal fluctuation. Three hypothetical life trajectories are depicted. A memory of environmental insult is mediated through epigenetic reprogramming in relevant tissues and brain regions.

In this way, epigenetic changes represent the molecular medium through which the environment influences phenotype. Numerous environmental factors have been linked to depression risk, including both low as well as high birth weight relative to gestational age [38,39]; early-life adversity, trauma, sexual abuse [40]; hormonal fluctuation [2–4]; and stress [41]. As will be outlined below, these environmental factors are capable of catalyzing epigenetic reprogramming at key genes that may lead to negative developmental outcomes and an increased susceptibility to MDD risk. As will be outlined for each case below, of critical importance is the developmental timing of environmental exposure in conferring disease risk. In this way, single instances of developmental insults or a combination of numerous insults over the course of development may lead to a vulnerability to stress, mediated by epigenetic mechanisms (Figure 14.1). The sections given below highlight the influence of each developmental insult on depression-relevant epigenetic changes.

Maternal diet and epigenetically mediated effects on neurodevelopment outcomes

One of the earliest possible time points that environmental influences may act on an individual is during gestation. One of the primary ways in which a mother may influence the developmental outcome of her offspring is through diet. In a study that introduced the concept of *in utero* programming of offspring outcome, a drastic increase in the rates of obesity was observed in male offspring over 50 years old that were born to mothers pregnant during the Dutch famine of 1944–1945 [42]. Although this example relates to obesity, it should not be overlooked that obesity is highly comorbid with depression [43], as are offspring both within the top tenth percentile and bottom tenth percentile for gestational

weight as a function of age [38]. Children in the bottom tenth percentile are referred to as small for the gestational age (SGA), while the top tenth percentile are large for the gestational age (LGA) [38]. SGA cases often arise due to placental blood flow restrictions or low maternal nutrition [38,44,45], and LGA cases may result from maternal obesity and gestational weight gain [46]. Maternal obesity may also restrict placental development [47], which has downstream consequences on the protection of the fetus from maternal stress hormones. Additionally, the placenta is an important source of serotonin, BDNF, and leptin, which play important roles in fetal brain development [38].

Both SGA and LGA exhibit consistent consequences in terms of dysregulated cell proliferation in the hypothalamus that affects key nutrient sensing pathways in the developing organism [38]. These may be linked back, in part, to alterations in placentally derived hormones, as leptin exposure of fetal hypothalamic neuronal progenitor cells in animal models led to a neuronal cell fate commitment, while insulin exposure led to a glial cell fate commitment as a function of hormone-induced DNA methylation changes [48,49]. Leptin and insulin also exert developmental influences on the VTA, a major source of dopaminergic neurons in the mesocorticolimbic reward circuit responsible for modulating hedonic feeding behavior [38,50]. Animal model SGA and LGA offspring exhibit deficits in spatial learning and memory that correlate with hippocampal volume reductions [51], which may be linked to altered BDNF signaling observed in both brain and placenta in both models [52]. Altered neuronal development in SGA and LGA offspring may be a consequence of maternal diet-induced epigenetic changes in the brain. Vucetic et al. [53] demonstrated that a maternal high-fat-diet induced DNA hypomethylation in key genes involved in mesocorticolimbic reward circuitry and the hypothalamus of the offspring including the dopamine reuptake transporter (DAT), μ-opioid receptor (MOR), and preproenkephalin (PENK) genes. Subsequently, Carlin et al. [54] showed that maternal dietary supplementation with methyl-donors reversed these previously observed high-fat-diet-induced incidences of weight gain and prefrontal cortical global hypomethylation. Such maternal diet-mediated epigenetic mechanisms may lead to neuroimaging alterations in the VTA such as hyperactivity observed in MDD [7,9].

Gestational consequences of maternal stress

In addition to the epigenetic effects of maternal diet on offspring neural development, there is evidence that epigenetic reprogramming of the offspring hypothalamic–pituitary–adrenal (HPA) axis may begin *in utero* and is influenced by the mother's state of mind and the associated downstream hormonal consequences that go with it. Growing evidence suggests that offspring of those women experiencing depression during pregnancy are at higher risk for various negative outcomes and psychiatric disorders. Davalos et al. [55], in a recent comprehensive review of the literature of the effects of antenatal depression on offspring outcomes, stated that such effects are both highly prevalent and understudied. One factor limiting progress in this field is a lack of consideration of the effects of antidepressant treatment vs. prenatal depression alone on offspring outcomes. For example, the incidence of maternal but not paternal depression was significantly associated with the incidence of autism spectrum disorder in a study of 4429 autism subjects and 43,277 matched controls [56]; however, it is unclear from this study if the effect was mediated purely by prenatal antidepressant use. As such, the most informative studies are those investigating treatment naive cases of antenatal depression and its effects on offspring. In the review by Davalos et al. [55], only 14 studies met these

stringent criteria. Numerous findings of low birth weight and preterm birth were observed in offspring of treatment naive antenatally depressed mothers. In some cases, these metrics were correlated with maternal cortisol during pregnancy. Of particular interest, however, are two studies identifying higher offspring cortisol levels and higher stress behavior in neonates born to antenatally depressed mothers [55]. Higher maternal anxiety was associated with lower offspring dopamine and norepinephrine levels in the offspring [57], both factors that can be downstream responses to alterations in estrogen and cortisol [58]. Maternal depression as well as antenatal scores on the Edinburgh Postnatal Depression Scale (EPDS) were associated with axial diffusivity and anisotropy in the right amygdala of their offspring [59]. Recently, a report by Hompes et al. [60] demonstrated that maternal anxiety and serum cortisol measurements taken during the antenatal period resulted in DNA methylation changes in the *NR3C1* gene in the cord blood of the offspring. Bromer et al. [61] showed that epigenetic variation of placental *NR3C1* was significantly associated with infant outcomes as measured by NICU Network Neurobehavioral Scales (NNNS), an indicator of early neurobehavioral outcome. Finally, placental epigenetic variation of the *11β-HSD2* gene was also associated with altered NNNS [62]. This gene is responsible for metabolizing maternal cortisol in the placenta to limit exposure to the developing fetus, further supporting the hypothesis that neurobehavioral outcomes may be mediated through cortisol-based epigenetic reprogramming [63]. Other studies suggest that gestational factors influence adult depression-like behavior through methylation of the *αCGRP* gene [64]. Cumulatively, these studies suggest that maternal anxiety and depression during the antenatal period may be linked to offspring outcomes such as the potential for altered HPA axis function and development of emotional processing of relevant brain structures through intrauterine environmental mechanisms. These developmentally acquired risks may in turn predispose to later-life depression risk.

It is important to consider how the above mechanisms may affect the implication of those heritability studies that have guided scientific efforts in psychiatric disease over the past 30 years. If maternal diet and mood states result in epigenetic reprogramming of HPA axis genes in the offspring, then there need not be a genetic component conferring risk in order to observe heritability. Those offspring subjected to maternal cortisol may in turn subject their own offspring to heightened cortisol when they are pregnant. This mechanism may represent only a portion of the potential risks to developing MDD. Although the amount of data to this effect is limited, an increase in the study of cord blood is likely to shed more light on this issue in the coming years.

Early-life adversity-mediated epigenetic reprogramming

In light of a growing literature on the effects of early environmental influence on epigenetic patterns in the brain, it is gaining acceptance within the scientific community that the effects of early environmental influences such as mothering behavior or early-life trauma can alter the long-term outcomes of cognitive development and stress response later in life [65]. Importantly, while extreme environmental insults such as childhood sexual abuse and assault represent the most widely studied and associated types of early-life adversity, more common and persistent stressors such as familial hostility, financial and food insecurity, and under-resourced schools and neighborhoods have also been linked with negative adult health outcomes such as MDD [65]. The molecular medium through which these long-term changes occur are epigenetic in nature, such as changes in DNA methylation reprogramming in the brain. Weaver et al. [66] conducted the seminal study demonstrating maternal care

influences on epigenetics and demonstrated that differences in arched back nursing and grooming of rat pups resulted in a DNA methylation change in the NGFI-A transcription factor binding site of the low-affinity glucocorticoid receptor (GR) gene, *NR3C1*, which is activated through the binding of stress hormone—corticosterone in rodents and cortisol in humans. DNA methylation changes in this gene resulted in altered stress response in the offspring rats later in life. Similarly, an early-life stress paradigm in mice demonstrated that DNA methylation induced increased expression of the arginine vasopressin gene in hypothalamic paraventricular nucleus neurons, which is critical for driving the neuroendocrine response to stress [67]. In humans, McGowan et al. [68] demonstrated an epigenetic association in the syntenic *NR3C1* exon 1F promoter in postmortem hippocampal tissue of suicide victims who experienced early-life trauma in the form of abuse. This study was the first to link this finding to humans. More recently, Katz et al. [69] performed a similar study in peripheral blood of abused and non-abused PTSD sufferers using a genome-wide microarray technology and identified distinct epigenetic profiles coinciding with the early environmental trauma. Pathway analysis of epigenetic variation induced by early-life trauma suggested an alteration of normal immune function. Importantly, this study was among the first to use genome-wide technologies to identify widespread epigenetic consequences across the genome in response to early-life stressors, demonstrating that the effects of early environmental influences are both widespread and detectable across tissues.

One implication of these findings is that the passage of HPA axis sensitivity and GR-mediated stress response phenotypes associated with depressive phenotypes could be influenced by mood disorders affecting the early postnatal period such as PPD. Only a handful of heritability studies have been performed in PPD; however, a systematic review of the literature suggests that treatment of PPD is insufficient to affect the long-term outcomes in child health [70]. This finding could be related either to an underlying genetic susceptibility or to risk conferred from the above intrauterine environmental mechanisms that are likely to be comorbid with PPD risk.

The epigenetic intersect of gonadal hormonal fluctuation and mood

Steroid hormones in addition to cortisol may have important implications for psychiatric disease. As mentioned above, rates of MDD double for women during the reproductive years, suggesting that there may be a role for the gonadal sex steroid hormones in depression etiology. From brain studies it is known that differential effects of androgens and estrogens contribute to neural development, affecting programmed cell death, cellular migration, synaptogenesis, axonal migration, and formation of sexually distinct neuronal circuits between males and females [71,72]. Other studies have shown that androgens are directly related to neurite arborization and estrogens are responsible for synapse formation and initiation of cellular communication [73]. Differences in the circulating hormone concentrations between sexes orchestrate the divergence of behavior and cognitive development in males through two related processes known as masculization and defeminization mediated by the estrogen receptor (ER) α and β, respectively [74].

Steroid hormones act as ligands to steroid hormone receptors such as the ER, a particular class of ligand-activated receptor known to alter chromatin structure through the recruitment of numerous interacting protein partners [75]. These interactors include co-repressor and coactivator complexes with chromatin-modifying capabilities, including histone acetyltransferases (HATs), histone methyltransferases (HMTs), and histone deacetylases (HDACs) [76,77]. The sex- and tissue-specific

differences in these co-regulator transcription levels have been suggested to result in variability in the level of response to hormone [78]. Periods of major hormonal rearrangement may therefore confer epigenetic changes capable of inducing epimutations with functional consequences. Joinson et al. [79] found that depressive symptoms among girls during mid-adolescence were more strongly influenced by breast stage than timing of menarche, implying that the female rise in depression during adolescence may be due to increasing levels of estrogen and may account for the gender difference in rates of depression at this stage.

One area experiencing significant progress toward understanding the epigenetic etiology of depression is female-specific mood disorders such as PPD. PPD occurs within 4 weeks following parturition according to DSM-IV criteria and follows a dramatic drop in the circulating levels of estradiol (E2) and progesterone (P4). Although depression risk is not predicted by serum levels of gonadal hormones in humans [80], rapid withdrawal from these hormones may be a key factor in establishing PPD. Bloch et al. [81,82] administered supra-physiological doses of E2 and P4 to a group of women with and without a history of PPD. Upon withdrawal from this hormone-mediated pseudo pregnancy, only the PPD history group experienced significantly depressed mood symptoms. These and numerous other studies suggest that alterations in estrogen signaling may underlie risk to PPD. Further hypotheses suggest that altered estrogen sensitivity may result in changes in HPA axis function and vulnerability to stress [58,82–84]. The release of corticosterone from the adrenal cortex into the rat hippocampus was found to be increased in female rats in proesterus rather than diesterus females or in male rats [85]. Similarly, ovariectomized (OVX) rats receiving E2 supplementation also exhibit higher corticosterone release, prompting Shansky et al. [58] to posit that estrogen may exacerbate the effects of stress-induced glucocorticoid release, a finding consistent with higher cortisol response in PPD cases during simulated pregnancy in humans [84]. Finally, women with PPD demonstrate increases in stress-induced cortisol and corticotropin-releasing hormone responses during pregnancy [86,87], resulting in reductions following parturition [84,88]. Many human studies have demonstrated that women with PPD exhibit lower baseline [89,90] or responsive [91,92] HPA axis function postpartum.

In a recent investigation in a prospective PPD cohort, Guintivano et al. [93] showed that women at risk for PPD exhibited an increased sensitivity to estrogen during pregnancy that manifested in epigenetic changes at hippocampally important loci. The effects of E2 on mood are believed, in part, to be localized to the hippocampus [94–98]. By modeling the effects of estrogen on DNA methylation change in the mouse hippocampus, Guintivano et al. [93] were able to predict PPD status using DNA methylation at syntenic genes using blood from a prospectively collected human PPD sample. These findings led to the identification of E2-responsive genes, *HP1BP3* and *TTC9B*, that can predict PPD status from antenatal blood DNA methylation with greater than 82% accuracy [93]. Epigenetic variation at loci in biomarker co-regulated gene networks was enriched for gene functions consistent with the known antidepressant functions of E2 in the hippocampus. Together, the data suggested that a difference in the epigenetic response to estrogen was mediating a risk for PPD and represented the molecular link between observations of estrogen sensitivity and the molecular neurobiology of the depressive phenotype.

The identified biomarkers also provided a suggestive link to an estrogen-mediated vulnerability to stress in the PPD population. The PPD predictive estrogen responsive biomarker *TTC9B* is hypothesized to inhibit HSP90-mediated trafficking of AMPA receptors critical for hippocampal LTP/LTD [93,99]. Interestingly, Schmidt et al. [100] demonstrated that differences in AMPA receptor

gene expression defined groups of mice as resilient or vulnerable to chronic social stress. This effect was abrogated by injection of an AMPA potentiator LY451646 [100]. AMPA receptors are linked to altered HPA axis responses, as injection of AMPA receptor agonists stimulated increases in hippocampal adrenocorticotrophic hormone (ACTH) levels [101]. In another study, plasma corticosterone and HPA axis function were linked to elevated levels of the GluR1 AMPA receptor subunit gene [102].These data represent another suggestive tie that the PPD biomarkers, which are indicative of E2 responsive epigenetic changes in PPD, may be markers of estrogen-induced changes on vulnerability to stress. These data are consistent with neuroimaging data implicating altered resting state functional connectivity in the postpartum period between frontal and hippocampal and amygdala regions in women experiencing PPD [103], suggesting that *TTC9B* and *HP1BP3* may represent trait as opposed to state markers critically involved in modulating PPD-relevant synaptic connections.

Inflammation

Inflammation has been implicated in MDD pathology, especially in the case of late-life-onset depression [104]. Microglial changes are observed in frontal cortical regions in older relative to younger depression-onset cases [105]. Inflammatory cytokines have been linked to both the stress response as well as alterations in hippocampal LTP [12,106,107], and higher levels of various proinflammatory cytokines have been found in older depressed men [108]. Furthermore, cytokines can activate the HPA axis [109], providing another suggestive link between inflammation and depression endophenotypes.

Elevations of many proinflammatory cytokines have been observed in non-late-life MDD as well as suicide [110], some of which have been posited to influence tryptophan availability to mediate suicidal behavior [111]. Furthermore, various studies have reported alterations in T-cell, granulocyte, and cytokine profiles in individuals exhibiting depressed mood [112,113]. Although numerous studies clearly outline the ways in which the immune system is under epigenetic control [114], these recent studies highlight that epigenetic variation may mediate behavior-associated inflammation. DNA methylation variation at numerous cytokine genes, including interleukin 1α (IL-1α), IL-6, IL-4, IL-8, and IL-10 and their associated transcription factors, was associated with childhood aggression [115], while epigenetic variation in cytokine promoter regions was associated with PTSD in military service members [116]. MDD cross-tissue epigenetic overlaps may be observed due to either cytokine-induced epigenetic reprogramming within target cells in both the CNS and the periphery or conversely by influencing the ratio of cell types in the sampled tissues and causing an identification of cell-type-specific epigenetic profiles due to proportional differences across disease phenotypes.

The effects of glucocorticoid system disruption in MDD

Approximately 70% of MDD cases occur within 6 months of experiencing a stressful life event [117], a statistic that highlights the importance of vulnerability to stress in developing depression. One of the most heavily studied genes involved in stress response is *NR3C1*, which encodes the stress hormone receptor GR. The importance of this system is further supported by the association of genetic variants within genes within the GR pathway such as *FKBP5*, which has also demonstrated a gene × environment interaction associated with depression [40,118]. As highlighted above, numerous

environmental influences etiologically linked to depression risk may alter the epigenetic status of *NR3C1*. Lower levels of GR may be responsible for brain activity endophenotypes observed in MDD. Cortisol levels are controlled via corticotropin-releasing hormone (CRF) from the hypothalamus, while various brain structures exhibit inhibitory and excitatory effects on hypothalamic CRF release. The hippocampus and rostral anterior cingulate cortex ultimately generate inhibitory effects on CRF through their connections with the perihypothalamic periventricular nucleus, while the amygdala inhibits this brain structure to enhance CRF release [119]. Importantly, altered levels of circulating glucocorticoids can create a positive feedback across these various brain structures leading to the hypercortisolemia [120] and relevant brain activity endophenotypes manifesting in the depressive phenotype. Cortisol increases lead to a GR-mediated decrease in rostral anterior cingulate cortex and hippocampal activity [121] and an increase in amygdala activity [5]. Additionally, dorsal prefrontal regions normally exerting an inhibitory influence on the amygdala are dampened by rising cortisol levels [5]. The net effect of these influences is an excitatory influence on CRF release that in turn leads to heightened cortisol release from the adrenal cortex. Importantly, the activity of this system is critically linked to the levels of the GR in various brain regions. *NR3C1* gene knockouts lead to drastically increased corticosterone levels, and hypothalamic conditional knockdown leads to normal baseline levels but exaggerated corticosterone release in response to stress [5]. These animal model observations are similar to phenotypes observed in suicidal youths, as indicated by increased cortisol release in response to stress and a failure to suppress the synthetic glucocorticoid dexamethasone. MDD-associated hypercortisolemia is mediated by a failure of the glucocorticoid negative feedback loop and an increased sensitivity to CRF and ATCH [12]. Stress exposure in early development can lead to amygdala hypertrophy and neuronal loss in the hippocampus and medial prefrontal cortex, and help to precipitate these phenomena in later life [122]. These results highlight that normal GR levels are critical for eliciting normal negative feedback systems on HPA axis function.

Evidence for altered GR function in MDD is further supported by Schlosser et al. [123] who assessed inhibitory response of prefrontal cortex (PFC)-mediated executive functioning using an emotional go/no go task designed to assess inhibitory response of PFC-mediated executive functioning. Glucocorticoid exposure enhanced inhibitory response in controls, but no effect was observed in MDD cases, leading the authors to conclude that the GR system is dysregulated in MDD. In a postmortem study of GR receptor expression in the brain, Alt et al. [124] demonstrated a significant decrease in the hippocampal-specific GR-1F variant in 12 MDD subjects; however, it was shown that the 1F promoter region previously reported to exhibit DNA methylation increases with child abuse was uniformly unmethylated in this sample. The observed GR-1F expression decreases were explained by a significantly reduced expression of the NGFI-A transcription factor known to bind to the 1F promoter in a DNA methylation-specific manner [124]. It is important to point out that, although dysregulation of the HPA axis may be an important contributor to MDD, there may be numerous mechanisms by which it is dysregulated. Early-life trauma may influence *NR3C1* DNA methylation, but not all MDD cases exhibit early-life trauma. The decreased NGFI-A transcription factor noted in the Alt et al. study may have critical implications on the HPA axis system through its modulation of GR expression, but may arise from other risk-inducing mechanisms such as maternal diet or hormonally induced epigenetic variation, for example. In this way, there may be multiple molecular phenocopies of complex psychiatric traits such as MDD. Importantly, environmentally induced variation in the HPA axis will lead to altered stress responses in individuals with MDD, which in turn may have additional epigenetic consequences that may contribute to monoamine and neurotrophic disruptions observed in MDD.

There is a rich animal literature demonstrating the myriad of epigenetic consequences in the brain in response to stress. These effects have been comprehensively reviewed by Stankiewicz et al. [41]; however, some brief examples are provided here. The hippocampus of Sprague–Dawley rats exposed to acute restraint stress demonstrates increased levels of histone H3 lysine 9 (H3K9) trimethylation and decreased histone H3 lysine 27 (H3K27) trimethylation, while chronic restraint stress exhibited increases in the activating histone H3 lysine 4 (H3K4) trimethylation levels [125]. H3K9 and H3K27 dimethylation levels were increased in the nucleus accumbens of C57BL/6ByJ mice subjected to social defeat stress, in mice vulnerable to the stress as opposed to those deemed resistant through performance differences in behavioral assays [126]. Using chromatin immunoprecipitation followed by microarray hybridization, the authors identified an enrichment for genes involved in cellular plasticity and inflammation [126]. Finally, the epigenetic patterns among behaviorally resilient mice were similar to vulnerable mice treated with the tricyclic antidepressant imiprimine [126]. Social defeat stress as well as isolation stress both influenced alterations in CREB-binding protein (CBP), the factor responsible for driving BDNF transcription [41]. Furthermore, BDNF is transcriptionally regulated by DNA methylation [127], while CBP has acetyltransferase activity. Cumulatively, the results suggest a potential stress-mediated epigenetic reprogramming of depression-related variation in the BDNF pathway that may be linked to MDD. This could contribute to environmental risks and their downstream epigenetic consequences.

Similarly, it is possible that variation in the 5-HT system may be a consequence of depression-related environmental risk factors outlined above. Recent investigations have pointed toward a role for DNA methylation variation in the *SLC6A4* gene promoter linking epigenetic variation of monoamine level regulation with MDD. In a study of 84 MZ twin pairs, Beck Depression Index (BDI) scores were significantly associated with 10 of the 20 promoter CpGs studied [128]. A notable advantage of the MZ twin design in this study is that genetic variation at this locus did not account for the observed associations with depressive symptomatology. A study of high and low stress groups in a cohort of nurses showed a significant effect of both work stress and workplace-induced depression on reducing *SLC6A4* promoter methylation [129]. In a longitudinal study of 222 patients, *SLC6A4* promoter methylation was significantly associated with poststroke depression at both 2 months and 1 year after stroke as well as worsening symptoms over the followed time period [130]. Another longitudinal study assayed T-cell and monocyte *SLC6A4* DNA methylation in adults with childhood aggression and showed that increasing promoter DNA methylation in both cell types correlated negatively with orbitofrontal cortex 5-HT synthesis levels measured using PET [131]. Consistent with a link between early environment and serotonin system phenotype, this effect was strongest in subjects with high childhood aggression. This is consistent with similar earlier reports that demonstrated a decrease in *SLC6A4* promoter DNA methylation with childhood sexual abuse [132–134]. Given its strong associations with both depression, anxiety, and depression brain activity endophenotypes, a stress induction model for epigenetic variation in key 5-HT system genes is an attractive candidate for future studies of epigenetics and depression-related brain imaging endophenotypes. An important caveat to consider is that the above studies in human populations were conducted in blood.

Epigenetic etiology and brain imaging endophenotype

Combining the field of neuroimaging and epigenetic analysis represents an attractive synergy of fields that may help to unravel the trait-based causal epigenetic variation identified in MDD from the

state-based changes downstream from disease etiology. Due to the tissue-specific nature of epigenetic patterns, the ability to combine epigenetic phenotypes and neuroimaging may be somewhat limited. The degree to which peripheral tissues such as blood or saliva may contain brain-relevant epigenetic signatures is a topic of continued study. Some initial reports using small sample sizes have investigated this issue. Davies et al. [135] performed methylated DNA immunoprecipitation (MeDIP) sequencing on blood, prefrontal cortex, and cerebellum from the same postmortem individuals and identified numerous regions of tissue-specific epigenetic variation; however, the authors noted that some inter-individual variation was reflected across brain and blood. Based on such evidence, the question becomes what mediates such cross-tissue epigenetic agreement across tissues? There are a number of possibilities. Petronis [136] has suggested that such cross-tissue agreement may result from a shared developmental origin prior to major tissue differentiation, such that inherited or early developmentally acquired epimutations may be reflected across primary germ layers. If this were the case, such epimutations may be expected to reflect those resulting from maternal diet or other intrauterine environmental epigenetic reprogramming events such as antenatal anxiety. Due to the epigenetic reprogramming inherent in the differentiation process, it follows that inherited or developmentally acquired epimutations would stand the best chance of being detected in regions not responsible for differentiation.

Alternative mechanisms may be responsible for cross-tissue correlations of epigenetic patterns such as those observed above in PPD. In this case, the implicated risk to disease involved a response to systemic hormone exposure capable of reprogramming epigenetic patterns. Peripheral detection of disease-specific epigenetic variation in peripheral blood is therefore a proxy marker of the deleterious hormonal influences on the brain in the at-risk group. A similar scenario is mirrored in the exposure to the stress hormone cortisol. Etiological insults resulting in altered cortisol response such as those observed in MDD may be expected to leave proxy epigenetic reprogramming in peripheral tissues, to some degree mirroring patterns that might be expected in the brain. These possibilities have been reviewed extensively by Gladkevich et al. [137]. Finally, inflammation-induced epigenetic variation observed in blood may mirror CNS epigenetic profiles assuming a common reaction to inflammatory cytokine profiles across tissues.

DNA methylome scans in MDD

Although there are numerous discrete examples of epigenetic alterations at candidate loci such as *BDNF*, *NR3C1*, and *SLC6A4* resulting from environmental influences, the genomic impact of the environment is not limited to single loci. For example, the effects of maternal care in the early postnatal environment on rat pups has been demonstrated to result in numerous DNA methylation changes in the chromosomal region proximal to the *NR3C1* [138]. With the advent of genome-wide epigenetic profiling technologies such as microarrays and next-generation sequencing, assessing post mortem brain tissue for epigenetic associations to depression becomes possible and will allow for the identification of novel loci where epigenetic variation may be of etiological relevance to the disease. Although important to the advancement of our understanding of epigenetics in psychiatry, the results of postmortem studies must be interpreted with caution, as numerous potentially confounding factors including medication status, mode of death, postmortem interval, brain pH, and cellular heterogeneity differences associated with the disease or treatment may lead to the identification of effects as opposed to etiological causes of the disease. To date, two studies have been performed using genome-wide technologies to investigate DNA methylation changes associated with MDD. In the first study, Sabunciyan et al. [139] used the

CHARM array to assess epigenetic changes in postmortem prefrontal cortical samples from the Stanley Medical Research Institute. This study did not identify any significant loci after correction for multiple testing, but it successfully validated 60% of the top nominally significant differences. Among the top hits was the *PRIMA1* gene, which functions to anchor acetylcholinesterase in neuronal membranes. The authors hypothesized that the observed 15% DNA methylation difference could result in increased cholinergic transmission in MDD. Subsequently, Guintivano et al. [140] analyzed 29 prefrontal cortical MDD samples and 29 matched controls on the Illumina® HumanMethylation450 BeadChip™ (HM450) in fluorescent-activated cell sorting (FACS)-separated neuronal and glial nuclei. The authors did not present a detailed depression-specific analysis of the data, as the primary thrust of that work was to generate a model for correction of cellular heterogeneity differences in a disease-independent manner. As a proof of principle of this work, the authors subsequently applied their brain cellular heterogeneity correction model, CETS, to the previously reported CHARM dataset, which resulted in the identification of significant genes surviving correction for multiple testing. Among these genes were longevity assurance homolog 2 (*LASS2*), which is associated with nerve repair [141], and cyclin-dependent kinase 16 (*PCTK1*), where overexpression in rats has been demonstrated to result in impaired spatial working memory and cognitive function [142].

A recent study has returned to the classical twin design to investigate epigenetics in MDD. Byrne et al. [143] used the HM450 array to assess peripheral blood DNA methylation in 12 MZ twins discordant for MDD and 12 non-discordant MDD pairs. The mean global DNA methylation was significantly different only in females, while 52.4% of probes on the microarray exhibited significantly higher variance in affected co-twins relative to their unaffected sibling, leading the authors to posit that affected twins may be subjected to environmentally induced epigenetic instability. Although additional factors such as the differential use of antidepressant medications in the affected co-twin group were partially addressed, the limited sample size of this study makes it difficult to draw concrete conclusions in this regard. One possibility is that MDD-associated alterations in immune cell profiles could account for the increased variation in the affected co-twins, though this specific hypothesis was not addressed in the study.

Returning to the issue raised above regarding non-shared environmental influence accounting for ~70% of MDD risk, how could MDD and epigenetic discordance among twins be accounted for in light of the theories presented in this chapter? One possibility is the differential intrauterine environmental influences of maternal mood and diet on twin outcomes. Approximately 70% of MZ twinning events occur after postimplantation day 4 and as such share a placenta and are termed monochorionic [144]. A well-known consequence of this is an uneven nutritional resource distribution to each twin through variable access to placental anastomoses for each twin. In theory, differential access to this placental support could lead to altered developmental trajectories such as those outlined in the maternal diet section of this chapter. More speculatively, in cases where excessive maternal cortisol may be influencing epigenetic reprogramming of key stress response genes in utero, such as *NR3C1*, differential access to placental connections may lead to discordance in the degree of epigenetic response to stress hormone, affecting later-life depression risk.

Conclusion

MDD and other depressive phenotypes result from a complex interplay of numerous risk factors. Heritability studies implicate a role for genetic factors, but to date few genetic variants have

demonstrated strong and consistent evidence for association to MDD. Heritability studies also implicate a large environmental component, and numerous environmental factors have been epidemiologically linked to depression risk. These include maternal high-fat or low-protein diet, high maternal anxiety or antenatal depression, early-life trauma, and stress. Risk for MDD may also be influenced by hormonal factors such as sensitivity to estrogen, as evidenced by increased rates of depression among females of reproductive age as well as the occurrence of female-specific mood disorders such as PPD. Finally, a susceptibility to neuroinflammation may result in instances of late-life depression. Importantly, the deleterious effects of these environmental risk factors interact with the genome through epigenetic mechanisms, leaving lasting DNA methylation and histone modification marks. Through the interaction with stress, this predisposition may lead to neuroconnectivity changes associated with disrupted emotional regulation and blunted reward circuitry. Although genetic variation is likely to exert some effect on MDD risk, the behavior-inducing changes of these epigenetic effects may lead to incidences of soft inheritance and require a careful reflection on the degree of genetic evidence for MDD.

The epigenetic effects of various environmental insults are only just beginning to be cataloged in a systemic manner with the help of genome-wide epigenetic profiling technologies. However, these techniques have their limitations, including a dependence on peripheral tissues that may not have relevance to the central nervous system in humans. Additionally, due to this tissue-specific nature of epigenetic patterns, subcellular heterogeneity within sampled tissues, such as varying lymphocyte proportions in whole blood or differing neuron to glia ratios in brain, will cause the identification of spurious disease-associated findings potentially resulting from diseased peripheral processes such as inflammation. The application and refinement of novel techniques recently generated to control for such heterogeneity will be necessary to identify the full range of etiologically relevant environmentally induced epigenetic variation in future studies.

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Glossary

Endophenotype A term used in genetic epidemiology to parse behavioral symptoms into more stable phenotypes with a clear genetic connection.

Epimutation Heritable defect involving epigenetic mechanisms of gene expression.

CHAPTER

15

The Epigenetics of Suicide: The Critical Impact of the Environment on Epigenetic Regulation in Suicide

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KEY CONCEPTS

- Childhood maltreatment is among the strongest predictors of stress-related psychiatric pathology and suicide.
- DNA methylation mediates lifelong neurobiological effects of early-life adversity.
- Animal and human studies reveal conserved epigenetic adaptations to early-life adversity.

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ABBREVIATIONS

5-HT Serotonin
5-HTT Serotonin transporter
ACTH Adrenocorticotropic hormone
AVP Arginine vasopressin
BDNF Brain-derived neurotrophic factor
CB1 Endocannabinoid receptor 1
CRE cAMP response element
CRH Corticotropin-releasing hormone
CSF Cerebrospinal fluid
DNMT1 DNA methyltransferase 1
ELA Early-life adversity
FKPB5 FK506 binding protein 5
GABA gamma-Aminobutyric acid
GAD1 Glutamate decarboxylase 1
GDNF Glial cell-derived neurotrophic factor
GR Glucocorticoid receptor
HPA axis Hypothalamic–pituitary–adrenal axis
HPC Hippocampus
LG Licking and grooming
MDD Major depressive disorder
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
mRNA Messenger RNA
NGFIA Nerve growth factor inducible A gene
OATL1 Ornithine aminotransferase-like 1
PFC Prefrontal cortex
POMC Proopiomelanocortin
PTSD Posttraumatic stress disorder
PVN Paraventricular nucleus of the hypothalamus
rRNA Ribosomal RNA
SAT1 Spermidine/spermine N^1-acetyltransferase
SMS Spermine synthase
TrkB Tyrosine receptor kinase type B
TSS Transcription start site
WHO World Health Organization

Suicide perspectives

Every year more than one million suicides are completed and more than 10–20 times more suicide attempts are committed [1,2]. In addition to the confusion and the pain generated by the loss of a loved one, suicide has major consequences on society. The World Health Organization (WHO) estimates the actual burden of suicide to be 20 million life-years and that, in 2020, suicide could be responsible for 2.4% of the total burden of disease [2]. Sadly, these statistics clearly show the important impact the phenomenon has on society but also highlight the poor understanding we have of the problem. The latter is particularly alarming considering that suicide can be avoided when effective treatments are delivered [3]. In this sense, it appears critical to develop new approaches to better define suicide and identify individuals at risk.

Suicide represents the extreme of a behavioral continuum defined as suicidal behaviors and classically occurs at the climax of a pathological process called the suicide crisis [4]. The complexity of suicide results mainly from the interactions of distal and proximal risk factors. Distal risk factors such as familial history of suicide, genetic and epigenetic factors, early-life adversity (ELA), and personality traits confer vulnerability to suicide, while proximal risk factors such as psychopathology, recent life events, hopelessness, and suicidal ideations more often trigger the suicidal crisis. It is no surprise that comorbid major depressive disorder (MDD) and substance abuse are among the strongest proximal risk factors. Estimates suggest that 50–70% of suicide completers die during an episode of MDD [5], and prospective follow-up studies of MDD suggest that between 7 and 15% of these patients will die by suicide [6–9]; however, only 10–15% of individuals affected by mental disorders (MDD, schizophrenia, and bipolar disorder) die by suicide [10]. In addition, mental disorders characterized by low impulsivity control such as substance abuse tend to be more strongly associated with suicide acts. In comparison,

depression tends to be more closely associated with suicidal ideation [11]. Importantly, age and other sociodemographic factors such as educational level, employment, and income modulate the impact of proximal factors on suicide risk [12–14].

On the other side of the spectrum are found several factors influencing suicide risk more distally. Personal and/or familial history of suicidal behavior is considered among the strongest predictive factors for suicide [15,16]. Genetic variations have also been consistently found to associate with suicide risk and account, in part, for the familial aggregation of suicide [17]. However, these appear to be independent of comorbid axis-I disorders [18] and rather seem to associate with the familial transmission of personality traits such as emotional and behavioral dysregulation, as well as cognitive impairments [13,19].

In addition to the above-mentioned factors, environmental factors and more specifically ELA events such as child abuse (sexual and physical) and parental neglect figure among the strongest correlates of depressive disorders [20,21] and suicide [22,23]. Although obtaining reliable estimates of the prevalence of childhood maltreatment is challenging, community studies suggest rates for all forms of maltreatment averaging around 10% in the general population. In contrast, up to 40% of suicide completers show evidence of childhood maltreatment [24,25]. In fact, ELA is estimated to increase the risk of suicide by 12 times [21,26]. Indeed, ELA has been associated with higher odds of self-harm [27–31], suicidal ideation [22,25], and suicide attempts [21,22,25,32–34]. Moreover, the prevalence of suicidal ideation and suicide attempts increases with the severity and intensity of the abuse [22,25,33]. Furthermore, ELA associates with increased comorbidity, earlier age of onset of depression, chronic course, and more severe depressive outcome [29,35–39]. The impact of ELA on suicide risk depends on the frequency of the abuse and the identity of the abuser, as an abuse perpetrated by close family member generates higher risks for suicidal behavior [22]. Indeed, close family members such as parents are essential for establishing appropriate emotional and behavioral patterns [40], and repeated acts of abuse by parental figures, or other close family members, may be indicative of an adverse environment to which the organism may respond by adapting key processes involved in emotional regulation and stress reactivity. Importantly, such adaptive processes may have pathological consequences and may confer susceptibility to develop suicidal behaviors when facing stressful and aversive situations.

While the relationship between ELA and maladaptive behaviors such as suicide is well accepted, the molecular mechanisms mediating these effects are still a matter of debate. Growing evidence suggests that epigenetic mechanisms may mediate the effects of stress on behavior. Epigenetics refers to the collective chemical and physical processes that program the genome to express its genes in a time- and cell-dependent manner. It includes posttranslational modifications of histone proteins, non-coding RNAs, and DNA methylation and hydroxymethylation. These mechanisms are capable of conveying information through meiotic and mitotic divisions in the absence of a change in the DNA sequence. The epigenome is responsive to developmental, physiological, and environmental cues. As such, epigenetics may explain how the environment regulates the genome and is well suited to mediate the effects of early environmental factors, potentially throughout the lifespan.

In this chapter, we present and discuss recent findings establishing a bridge between early-life adversity and increased risk for suicide by focusing mainly on DNA methylation, the epigenetic mark that received by far the most interest in relation to suicide. With this in mind, we propose a model in which the neurobiological consequences of early-life adversity build into long-lasting and stable behavioral patterns characterized by certain personality traits that in turn may increase suicide risk.

The molecular consequences of early-life adversity: Transcriptional regulation by epigenetic mechanisms

From a molecular point of view, it has been challenging to conceptualize how events occurring early in life can induce long-lasting behavioral modifications. Although these behavioral changes have to pass through modifications of gene expression, they also have to be long lasting. Thus, if epigenetic mechanisms are believed to mediate the influence of the environment on the genome, the precise molecular mechanisms are still obscure. Nevertheless, major breakthroughs into this critical issue have been made in animal studies and, more recently, from translational studies performed in human postmortem brains.

It stands to reason that intense and prolonged stressors occurring early in life, such as child abuse, indicate to the individual that the environment they are in is unsafe. These stressors extensively and abnormally activate the hypothalamic–pituitary–adrenal (HPA) axis, leading to long-lasting functional molecular alterations that have been reported both in humans and animals. A series of elegant groundbreaking studies focusing mainly on specific key players in HPA axis activity provided major insights into the molecular mechanisms by which early-life stress induces long-lasting behavioral alterations. These studies are discussed below.

Glucocorticoid receptor

One of the first studies addressing this issue focused on the transcriptional regulation of the glucocorticoid receptor (GR) in the brain of offspring from low licking and grooming (LG) mothers. This work shows that pups raised by low LG mothers exhibit lower hippocampal expression of the hippocampal-specific variant of GR ($GR1_7$) compared with those raised by high LG mothers [41] and was later on complemented by findings showing similar alterations in various other variants of GR ($GR1_5$, 1_6, 1_8, 1_9, 1_{10}, and 1_{11}) [42]. This was associated with significant higher DNA methylation levels in respective promoters, including regions overlapping binding sites for transcription factors (e.g., NGF1A) known to regulate GR expression. More recently, the analysis of a 6.5 million base-pair region centered on this genomic region further strengthened the notion that early-life stress induces behavioral changes by affecting the epigenetic control of genes. Indeed, Suderman and colleagues showed that poor maternal care associates with hundreds of parallel DNA methylation changes in rats [43]. These adaptations preferentially affected promoters, as evidenced in the cluster of protocadherin genes, and followed a nonrandom, discontinuous pattern across large genomic regions [43].

Interesting molecular correlates can be found in human victims of child abuse. Indeed, translational studies, aiming at elucidating the molecular counterpart of ELA in the human brain, found lower levels of both GR_{total} and $GR1_F$ (the human homolog of $GR1_7$ in rats) expression in the hippocampus (HPC) of abused suicide completers compared with non-abused suicides and controls [44]. This was also associated with sites of hypermethylation in the promoter region of $GR1_F$, one of which was found within a NGFIA binding site. Functional experiments showed that methylation at this precise site represses the binding of NGFIA to DNA and decreases the transcriptional activity of the promoter, providing a potential explanation for the lower expression of GR found in the brain of abused suicide completers. Follow-up work showed that other GR variants ($GR1_B$, 1_C, and 1_H) are also downregulated in the HPC of abused suicide completers, and this was associated with several sites of differential methylation in respective promoters [45] (Figure 15.1).

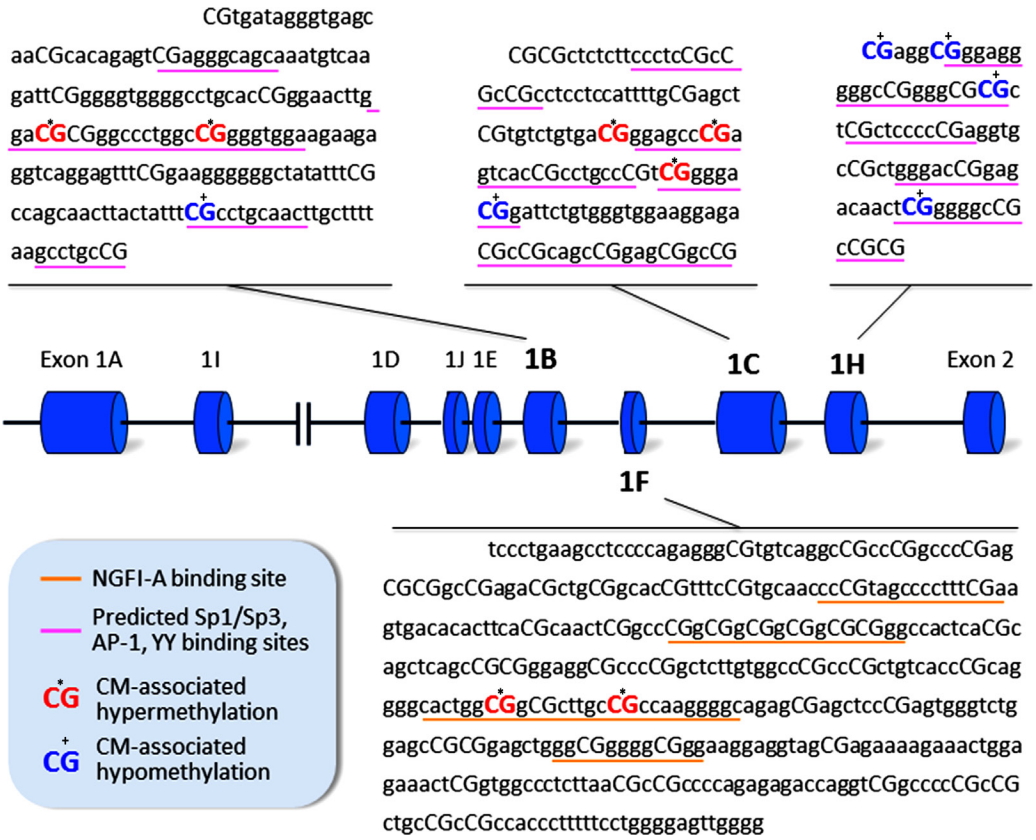

FIGURE 15.1

Childhood maltreatment modifies DNA methylation pattern at the human glucocorticoid receptor (GR) gene in postmortem brain hippocampal tissues. Childhood maltreatment associates with both increases (asterisk) and decreases (cross) in levels of DNA methylation across several CpG sites in the various 50-untranslated exons of the human GR gene. DNA sequences from promoter regions of GR exons 1B, 1C, 1F, and 1H are shown. Underlined sequences correspond to binding sites of several transcription factors, according to: (1) *in silico* prediction for Sp1/Sp3, AP-1, and YY; and (2) both *in silico* and *in vitro* studies for NGFI-A. The functional transcriptional relevance of DNA methylation levels has been assessed *in vitro* for exons 1F [44], but not yet for exons 1B, 1C, and 1H [45].

Adapted from Lutz PE, Turecki G. DNA methylation and childhood maltreatment: from animal models to human studies. Neuroscience 2014:264;142–56.

These findings were, more recently, supported by a series of studies investigating whether similar changes can be found in the blood of ELA victims. Indeed, the infants of mothers reporting intimate partner violence during their pregnancy showed higher levels of methylation in the promoter of $GR1_F$ compared with those born from normal mothers [46]. Another study reported correlation between $GR1_F$ promoter methylation levels and parental loss, child maltreatment, and parental care in leukocytes from healthy adults [47]. Furthermore, DNA methylation levels in $GR1_F$ promoter were shown to

be positively correlated with childhood sexual abuse, with its severity and the number of maltreatment types in individuals with MDD, and with repetition of severe types of abuse in patients with bipolar disorders [48].

At the intracellular level, FK506 binding protein 5 (FKBP5) is a chaperone protein that inhibits GR ligand binding and translocation of the GR–ligand complex to the nucleus. Several glucocorticoid response elements are present at the FKBP5 gene, so expression of FKBP5 is stimulated by glucocorticoids and represents an intracellular short negative feedback loop. Independent groups have reported that genetic polymorphisms in FKBP5 (including haplotypes rs1360780, rs9296158, rs3800373, and rs9470080) interact with a history of ELA to predict posttraumatic stress disorder (PTSD), suicide attempts, and depression in adulthood [49–51]. Recently, an epigenetic mechanism was described at a glucocorticoid response element in the FKBP5 intron 2 (rs1360780) that may account for such an interaction [52]. Accordingly, *in vitro* experiments suggested that both baseline and corticosteroid-induced expression of FKBP5 are increased in carriers of the risk allele (rs1360780 A) compared with carriers of the protective allele (rs1360780 G). Using an elegant chromatin conformation capture approach, the authors were able to demonstrate that the risk allele, which creates a new TATAA box distant from the FKBP5 transcription start site (TSS), potentiates and favors recruitment of the RNA polymerase II through a tridimensional interaction among intron 2, intron 7, and the TSS. Noteworthy, decreased DNA methylation levels were measured in intron 7 of the gene as a function of ELA (in peripheral blood) and likely resulted from this transcriptionally active conformation. Therefore, in risk carriers, FKBP5 negative feedback onto GR activity is potentiated, impairing the ability of glucocorticoids to retro-control the HPA axis and ultimately potentiating the effects of ELA-associated stress. These interesting data emphasize the need to combine analysis of DNA methylation and other measures of chromatin structure across large genomic regions to decipher complex, long-range epigenetic interactions.

Corticotropin-releasing hormone

In addition, other studies have provided evidence suggesting alterations of other key constituents in the activity of the HPA axis in suicide but also following chronic stress. For example, higher plasma adrenocorticotropic hormone (ACTH) and cortisol levels [53] and lower cerebrospinal fluid (CSF) oxytocin levels [54] have been reported following stressful conditions. Elevated CSF corticotropin-releasing hormone (CRH) levels [55] are also common features in suicide. Indeed, suicide completers exhibit hyperactivity of CRH neurons in the paraventricular nucleus of the hypothalamus (PVN) as suggested by increased CRH messenger RNA (mRNA) levels and increased numbers of CRH-positive neurons [56–58]. Moreover, several brain regions from suicide completers have been characterized with decreased CRH binding sites [59], altered CRH receptor-type ratios [60], and elevated CRH immunoreactivity and mRNA levels [61,62].

Although no study in humans has yet assessed the molecular effects of ELA on the epigenetic regulation of these genes, recent studies in stressed mouse revealed complex epigenetic alterations in the regulation of CRH [63] that could also be taking place in humans. Indeed, higher CRH expression in the PVN of chronically stressed mice was associated with significant hypomethylation in a cAMP response element (CRE) binding site within the CRH promoter, the importance of which was further confirmed by luciferase assays showing that mutating a single base in the CRE binding site substantially reduced the cAMP-induced CRH promoter activity [63]. Interestingly, chronic treatment with the tricyclic imipramine attenuated the behavioral, transcriptional, and epigenetic changes induced by social

stress [63]. Moreover, similar findings have also been reported in mice with a different stress paradigm [64], supporting the validity of these findings.

It is known that mothers suffering from stress-related psychopathology expose their progeny to an increased risk of negative mental health outcomes in adulthood [65–67]. Thus, Franklin and colleagues used a series of maternal manipulations to assess the transgenerational impact of stress on behavior and epigenetic control of gene expression. Interestingly, the authors report differential DNA methylation in critical genes including methyl-CpG-binding protein 2 (MeCP2), CB1 (the main endocannabinoid receptor in the brain), and the CRH receptor 2 (CRH-R2) in both germ lines and brain. Of note, similar transgenerational transmission of behavioral deficits has been recently documented in the chronic social defeat paradigm applied to adult mice, although results were contrasting [68], suggesting that early life but not adulthood may represent a sensitive period for epigenetic changes to occur in the germline and to be transmitted in the offspring.

Arginine vasopressin

Similarly, altered expression of several neuropeptides, including the arginine vasopressin (AVP) and proopiomelanocortin (POMC) peptides, was reported in several brain regions of suicide completers [61,69]. In addition, a significant positive correlation was reported between plasma AVP and cortisol levels in depressed individuals, and AVP levels were elevated in subjects who had attempted suicide [70].

Here, again, although no human study has yet highlighted molecular alterations in the epigenetic control of AVP expression in human brain, animal studies have begun to address the question. Indeed, AVP expression in the PVN (and POMC) has been associated with stress-coping alterations in adult offspring separated from their mothers [71]. The mechanisms underlying these modifications require the activity of an enhancer element found outside the body of the gene [71,72]. Methylation levels at numerous sites within this enhancer were shown to be significantly downregulated in stressed mice and inversely correlated with AVP expression.

Overall, these results focusing on genes known to be significantly involved in the function of the stress axis, and combining both human and animal data, suggest that early-life experiences regulate lifelong stress reactivity through similar epigenetic processes across species. Thus, in the context of the HPA axis function, in which several genes interact to control the activity of the axis, these effects may appear to be particularly functionally relevant. This is especially so considering that these effects may not be restricted only to the activity of the HPA axis but also other important behavioral and cognitive functions. It is indeed believed that such alterations may be critical in the development of suicidal behavior [73]. In the following section, we will continue exploring epigenetic alterations that have been reported in the brain of suicide completers and may be relevant to the etiology of suicide.

Neurotrophic factors

In addition to members of the HPA axis mentioned in the previous section, neurotrophic factors, most notably brain-derived neurotrophic factor (BDNF) and its receptor, tyrosine receptor kinase type B (TrkB), represent another group of genes that have been consistently investigated in the context of psychiatric disorder and stress [74]. Considering the wealth of data on the role of BDNF in anxiety and depression in both animal models and humans [75], it seems clear that this neurotrophic factor has a role in mediating stress responses in the brain, and epigenetic dysregulation of this gene is likely to

affect emotional maturation and to increase suicide vulnerability. Most human studies on BDNF in relation to mood disorders have focused primarily on polymorphisms in the BDNF gene, in particular the Val66Met polymorphism. For example, lower serum BDNF levels have been reported in carriers of the BDNF Met allele exposed to childhood abuse, although exposure to negative life events itself was sufficient to decrease serum BDNF levels independent of genotype [76]. In addition, evidence from human studies suggests that decreased BDNF expression, at least in blood (lymphocytes and platelets), is associated with suicide attempts and anxiety-related traits [77]. However, during the last decade, interest in BDNF switched toward the relationship among BDNF expression levels, epigenetic regulation, and response to stress in rodents. Chronic social stress in mice and exposure to abusive mothers and traumatic stress in rats were shown to decrease the expression of BDNF transcripts III and IV in the hippocampus [78,79] and the prefrontal cortex [79,80], respectively. Although similar, these transcriptional alterations are induced by different epigenetic mechanisms. In fact, while chronic stress in mice increases hippocampal H3K27 dimethylation levels at the BDNF promoter and compacts chromatin [78], traumatic stress in rats was shown to alter DNA methylation patterns in distinct regions of the hippocampus [79]. Similarly, exposure to abusive mothers in rats was associated with site-specific hypermethylation in the promoters of transcripts IV and IX in the prefrontal cortex [80].

Based on the previous evidence, our group provided evidence suggesting that antidepressants promote open chromatin structure (lower H3K27me3 level) in the promoter of BDNF in the prefrontal cortex (PFC) of suicide completers [81]. In addition, hypermethylation at four sites within the promoter of BDNF IV was reported in the Wernicke area of suicide completers which was inversely correlated with BDNF expression levels [82]. Follow-up studies in the blood of depressed subjects revealed higher BDNF expression in the blood of treatment responders (citalopram) compared with non-responders [83]. Interestingly, H3K27me3 levels were inversely correlated with both BDNF IV expression levels and the severity of symptoms. In another attempt to translate animal findings to humans, methylation states of the BDNF gene were examined in peripheral blood leukocytes from borderline personality patients. Results indicated that DNA methylation in the BDNF promoter increases as a function of the number of childhood traumatic experiences in these patients [84]. Of note, this study found no correlation between DNA methylation state and expression level of BDNF in the plasma; thus, the functional relevance of this finding is unclear.

Tyrosine receptor kinase B, the BDNF receptor, has also been strongly associated with mood disorders. For example, lower TrkB expression has been reported in the PFC of depressed subjects [85,86], and antidepressant treatment has been shown to increase its expression in cultured astrocytes [87]. The astrocytic variant of TrkB, TrkB.T1, was also shown to be downregulated in the PFC of a subset of suicide completers. Interestingly, an inverse correlation was found between TrkB.T1 expression and promoter methylation, from which, two hypermethylated sites were found in suicide completers [88]. In addition, suicide completers with low TrkB.T1 expression showed enrichment of H3K27 methylation in the TrkB promoter [89], suggesting the presence of a dual epigenetic control over the expression of the astrocytic variant of TrkB. Interestingly, recent data showed that mice overexpressing the TrkB.T1 variant are more susceptible to chronic social stress than wild-type mice by exhibiting consistent social avoidance [90]. Together, these data suggest that epigenetic changes in TrkB.T1 promoter could define the vulnerability to chronic social stress and possibly to early-life adverse experience. More recently, interesting data have suggested that the epigenetic regulation of the glial cell-derived neurotrophic factor (GDNF) combined with genetic background and environmental factors may contribute to the behavioral responses to stress in different strains of mice [91]. Indeed, low GDNF levels, as well as a closed

chromatin conformation in GDNF promoter (less histone acetylation and methylation), have been associated with depression-like phenotype in a stress-susceptible mice strain (BALB) that faced chronic stress. This was correlated with a higher level of methylation in the GDNF promoter that promoted the binding of the transcriptional repressor MeCP2. Although some of these differences were also found in the "resilient" strain, findings were more consistent in the stress-susceptible strain [91], suggesting that the behavioral effects of chronic stress may pass through epigenetic modifications.

Other epigenetic alterations in suicide brains

In addition to the findings discussed above, studies have focused on other functional systems, which had been implicated in depression and suicide. Among these systems, genes involved in protein synthesis such as ribosomal RNA (rRNA), polyamines, and GABAergic and serotonergic systems are noteworthy.

Ribosomal RNA gene

Having the role of decoding mRNA into amino acids, ribosomal RNA (rRNA) is a bottleneck structure for protein synthesis, allowing adequate cell function depending on the cell needs. The expression of multiple copies of rRNA genes present in the genome has been shown to be epigenetically regulated both in mice [92] and humans [93,94]. Indeed, promoters of active rRNA gene copies are completely unmethylated, while inactive copies are almost fully methylated [93]. Importantly, this epigenetic control appears to be dysregulated in the hippocampus of abused suicide completers [95]. Abused suicide completers exhibited smaller rRNA expression levels associated with increased methylation in 21 out of 26 CpGs found within the rRNA promoter compared with controls. Interestingly, these alterations seem to be specific to the HPC, as no group difference in rRNA methylation pattern was found in the cerebellum.

Polyamine system

Polyamines are ubiquitous aliphatic molecules involved in cellular functions, including growth, division, and signaling cascades [96,97]. The polyamines also play a major role in the regulation of stress [98,99], being dependent on activation of the HPA axis and increased concentrations in circulating glucocorticoids [96]. Furthermore, emergence of the characteristic adult polyamine stress response correlates with cessation of the hyporesponsive period of the HPA axis system [100]. Previously, spermine synthase (SMS), spermidine/spermine N^1-acetyltransferase (SAT1), and ornithine aminotransferase-like 1 (OATL1) expression has been shown to be altered in the limbic system of suicide completers with a history of depressive disorders [101,102]. Follow-up studies have begun to implicate epigenetic mechanisms in this dysregulation of the polyamine system, with changes in both DNA methylation levels [103] and microRNA (miRNA) posttranscriptional regulation [104].

GABAergic system

The GABAergic system has been the focus of many studies in postmortem brain samples of psychiatric patients, particularly individuals with histories of depression [105–107], schizophrenia, or bipolar

disorder, many of whom died by suicide [108–111]. For example, reductions of reelin and glutamate decarboxylase 1 (GAD1) mRNA [108] and an increase in DNA methyltransferase 1 (DNMT1) expression [112,113] were previously reported in postmortem brains of schizophrenia and bipolar subjects who died by suicide. Consistently, promoter hypermethylation was reported for both genes in accordance with the methylating role of DNMT1 [114,115]. These findings agree with results from Poulter and colleagues [116], who examined the expression of DNA methyltransferases as well as $GABA_A$ receptor α1 subunit in the brain of suicide completers. Hypermethylation in α1 subunit promoter was identified in the prefrontal cortex of suicide completers which was negatively correlated with DNMT3b protein expression. Besides DNMT3b, DNMT1 and DNMT3a levels have also been found to be altered in the limbic system and brain stem of suicide completers [116]. Although in this study there was no report of histories of ELA, these findings fit well with more recent animal data [117]. Indeed, pups raised by low LG mothers were shown to have lower GAD1 hippocampal expression, which associated with promoter hypermethylation and lower levels of H3K9ac compared with pups raised by high LG mothers. The authors also reported higher DNMT1 hippocampal levels. Functional assays revealed that the transcription factor NGFIA binds the GAD1 promoter in order to increase GAD1 expression. Consequently, these results suggest that, similarly to the regulation of GR in rat hippocampus, GAD1 expression is modulated by maternal behavior via epigenetic mechanisms involving DNA methylation interfering with the binding of activating transcription factors and by chromatin modifications [117].

Serotonergic system

The serotonergic system is a neurotransmitter system of great importance in psychiatry that has been extensively investigated in depression and suicide. Lower concentration, neurotransmission, and reuptake of serotonin and its metabolites are risk factors for suicidality and major depression [118,119]. Among the various serotonergic receptors, particular attention has been given to $5\text{-}HT_{2A}$ as an important candidate in association studies of suicidal behavior [120,121]. One of the genetic variants most commonly investigated is the 102 C/T polymorphism located in exon 1 [122,123]. Methylation in the C allele variant in this polymorphism, as well as $5\text{-}HT_{2A}$ expression, had previously been correlated with DNMT1 expression, in both the brain and leukocytes of healthy subjects [124]. Methylation was increased in leukocytes from patients reporting suicidal ideation, but a trend toward hypomethylation was reported in the prefrontal cortex of suicide completers carrying the C-allele [125], suggesting that methylation levels may be different in individuals who committed suicide and those who are planning suicide.

Another serotonergic gene that received considerable attention in the context of ELA-induced epigenetic regulation is the serotonin transporter (5-HTT) [126]. In peripheral blood mononuclear cells from Rhesus macaques [127], an interaction was reported between early attachment pattern and the methylation state of the 5-HTT promoter, whereby increased methylation in this genomic region associated with increased reactivity to stress in maternally deprived, but not in mother-reared, infants. In human studies, transformed lymphoblast cell lines derived from subjects recruited through the Iowa Adoption Study were used [128]. Interestingly, a significant association emerged between sexual abuse alone and overall DNA methylation in the 5-HTT gene promoter, among females. Building on this work, the authors explored another, non-overlapping cohort of female subjects [129] and confirmed the hypermethylation in the 5-HTT promoter with ELA victims. Furthermore, epigenetic patterns in this gene also associated with the emergence of antisocial personality disorder in adulthood. While these studies should be interpreted with caution, the same investigators subsequently reported that parental history of

psychopathology interacts with ELA to predict the intensity of 5-HTT DNA methylation changes and the risk of antisocial personality disorder [130]. Finally, they also explored DNA methylation across other, non-promoter regions of the 5-HTT gene, and reported two additional sites of differential methylation as a result of ELA [131], although expression of the 5-HTT was unaffected. Results from these series of peripheral studies focusing on candidate gene are encouraging and clearly suggest that epigenetic marks may serve as biomarkers of early stressful life experiences and the risk of psychopathology.

Investigating genome-wide DNA methylation patterns

Overall, environmental factors seem to epigenetically regulate genes involved in key regulatory processes, such as the HPA axis and neurotropic factors. This finding is supported by numerous other studies looking at genes involved in neurotransmission [116,123,125,132–135], polyamines [136,137], and protein synthesis [95], for example. The contribution of epigenetic factors to the regulation of numerous candidate genes has now been demonstrated, but there is a real need for large-scale comprehensive studies assessing genome-wide epigenetic patterns in the context of different environmental factors. A few of these studies recently came out with interesting findings suggesting that environmental factors, while targeting critical genes, may also induce genome-wide epigenetic reprogramming.

Our group recently assessed the impact of child abuse on genome-wide DNA methylation signatures in gene promoters [138]. In this study, we compared the hippocampal DNA methylation patterns of 25 suicide completers with a severe history of child abuse (sexual and physical) to those of 16 healthy controls. We identified hundreds of sites differentially methylated, both hyper- and hypomethylated, in the hippocampus of severely abused suicide completers. Interestingly, DNA methylation levels in gene promoters were inversely correlated with gene expression at a genome-wide level, supporting the globally repressive role of DNA methylation in promoters, as reported by other groups [139,140].

Similar observations have also been made in suicide completers, the data showing commonalities with the abused suicide group, but also substantial differences [141]. The impact of abuse becomes obvious when assessing the gene functions enriched with differential methylation. Indeed, differential methylation in the abused suicide group is enriched in genes related to cellular plasticity, while learning and memory genes were particularly affected in suicide. This suggests that intense early-life adversity may induce long-lasting alterations that may not be found in the brain of suicide completers for which early-life adversity was adjusted.

Similar findings have also been reported in the blood of PTSD patients [140]. However, in this study, the analysis of DNA methylation levels was not restricted to promoters but rather to 14,000 CpGs across the genome. Functional annotation revealed an over-representation of differentially methylated CpGs in genes related to immune functions, supporting the notion that early-versus later-life stress may recruit specific epigenetic and psychopathological processes, which may be translated into the development of differentially methylated CpGs. This being said, a previous study performed in the PFC of psychotic and bipolar patients reported numerous sites of differential methylation that were enriched in various functions such as glutamatergic and GABAergic neurotransmission, brain development, and response to stress [139]. Importantly, these studies, conducted in different tissues (blood versus brain) and brain regions (HPC versus PFC), globally suggest that stress-induced epigenetic adaptations are region specific, consistent with the emerging notion of epigenetic heterogeneity across tissues [142] and cell types [143,144].

Finally, the first genome-wide studies of DNA methylation changes in peripheral tissues following ELA and related adverse experiences were recently reported. Smith and colleagues tested correlations between DNA methylation in blood with histories of ELA, PTSD, and total life stress exposure [145]. Differential methylation in five genes and one gene associated with PTSD and total life stress, respectively, but no significant finding was reported for ELA. In children exposed to institutional placement, compared with children raised by their biological parents [146], more than 800 differentially methylated genes (among which 90% are hypermethylated) were detected, including genes implicated in cellular signaling, in blood immune responses and brain function. In saliva samples from children having experienced severe enough ELA to warrant out-of-home placement [147], more than 2600 CpG sites with differential methylation levels were reported in abused children compared with a control group of children that had no experience of maltreatment and no psychiatric illness. In line with previous results in the brain, these studies suggest that the experience of ELA and related early stressful experiences have profound, genome-wide epigenetic consequences in peripheral tissues. In the future, assessing epigenetic plasticity in both central and peripheral tissues of the same individuals, through similar technologies, will be required to explore their potential relationships. A recent study addressing this daunting task in relation to ELA was able to provide evidence for some small overlap between epigenetic changes in the prefrontal cortex and circulating lymphocytes of rhesus monkeys [148].

Toward an integrative model of stress-induced psychopathology

Suicide is obviously a complex phenomenon that results from different etiological pathways and, as such, no single neurobiological mechanism can explain alone the complexity of the suicide process; however, a sizable proportion of individuals with suicidal behaviors have suffered from child abuse, and these individuals may share common characteristics. The findings presented in this chapter support this view and provide several new avenues of investigation for future work targeting previously unexpected functional pathways that may be involved in the etiology of suicide and which may be specifically modified by ELA (Figure 15.2).

In light of what has been described in the previous sections, ELA is known to induce long-lasting molecular changes in the brain by interfering with the epigenetic regulation of genes. These changes have been shown to affect in particular the HPA axis [41,42,44–48] and neurotrophins [79,80,82,88,89], but also cellular plasticity [149], learning and memory [150,151], and the immune system [140]. In parallel to these molecular events, ELA also induces brain structural changes that may associate with sustained neurobiological alterations. Indeed, ELA has been associated in various brain regions with significant changes in gray matter volume, including the anterior cingulate gyrus, dorsolateral, and medial PFC [152,153]; superior temporal gyrus [154]; amygdale [155]; hippocampus [155,156]; and corpus callosum [156]. Functionally, these structural changes are expected to have a significant impact on the development of personality traits such as emotional and behavioral dysregulation, as well as cognitive impairments that have been substantially associated with increased risk for suicidal behaviors [13]. Of particular interest are results from longitudinal studies suggesting that developmental trajectories of anxiousness or disruptiveness (impulsivity and aggressivity) are highly predictive of suicidal behaviors in adulthood [157–161]. Furthermore, ELA has been associated with cognitive impairments, such as decision-making and problem-solving deficits [161–164], which are expected to increase the vulnerability toward suicidal behaviors [165–169]. For instance, exposure to stressful situations has

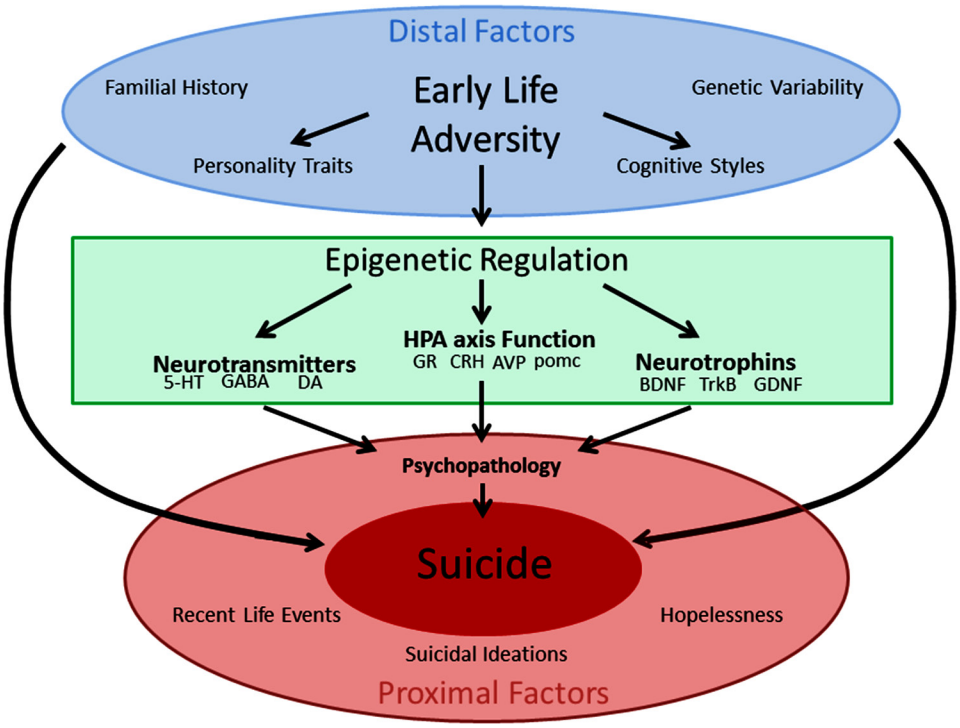

FIGURE 15.2

Schematic model of the mechanisms by which early-life adversity increases risk for psychopathology and suicide. Early-life adversity interferes with the epigenetic control over the expression of genes coding for components of the HPA axis and neurotrophic factors. This, in turn, leads to the development of high levels of anxiousness and impulsivity. These behavioral traits increase vulnerability for suicidal behavior by acting as distal risk factors increasing suicide risk when in combination with precipitants such as depressive psychopathology and substance disorders. *Abbreviations*: 5-HT, serotonin; AVP, arginine-vasopressin; BDNF, brain-derived neurotropic factor; CRH, corticotropin-releasing hormone; DA, dopamine; GABA, gamma-aminobutyric acid; GDNF, glial-derived neurotrophic factor; GR, glucocorticoid receptor; POMC, proopiomelanocortin; TrkB, tyrosine receptor kinase B.

been shown to impact decision-making and problem-solving skills in individuals with suicidal behaviors displaying dysfunctional responses to stress [14,170–173].

With this in mind, one may hypothesize that the combination of anxious and impulsive personality traits with cognitive impairments and dysregulated stress reactivity will mediate the relationship between ELA and behavioral alterations, including suicide. This model, however, is simplistic in the way that it oversimplifies complex behaviors but may, in fact, explain a large part of the vulnerability to psychopathology in individuals who suffered ELA. One can imagine that, in the context of daily life stress, individuals who developed these personality and cognitive traits following ELA may be more at risk to develop suicidal behavior.

Concluding remarks

The findings presented in this chapter generate several interesting questions but also raise significant limitations that will have to be addressed in future studies. For example, one key question is whether similar DNA methylation alterations can also be found in gene bodies. Indeed, most of the findings reported here refer to gene promoters because of the regulatory roles these regions have on gene transcription. However, there is a growing body of evidence suggesting that other regions as well may be involved in transcriptional regulation [174]. It will also be important to consider other epigenetic mechanisms such as histone modifications [175,176], hydroxymethylation [177], and other non-CpG-based modifications [178,179] that are likely to interact in the regulation of gene expression. Another important point is cell specificity. Indeed, although epigenetic mechanisms are known to be cell specific, very few of the findings reported in this work considered that fact. With the development of more sophisticated tools, future studies should begin addressing this important question.

Finally, most of the studies to date have focused on males, but evidence suggests that females are more susceptible to the effects of stress and, as such, one may expect to find similar but also different epigenetic alterations in females [180]. Moreover, previous studies showed that stress induces dramatic sex-specific changes in gene expression [181], and it has been suggested that these changes may reflect sex differences in the reorganization of neural circuitry in response to stress.

Science and technology evolve at a fast pace, and these questions will most likely find answers sooner rather than later. At the same time, these answers may generate new questions that will require the development of new innovative experimental strategies. At this point, our understanding of mental health is limited by our understanding of the genome itself. This is why new innovative multidisciplinary approaches are now becoming essential to improve our understanding of mental health and disease.

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Glossary

Arginine vasopressin Vasopressin that contains arginine, an amino acid, as in humans and most other mammals. It is a polypeptide synthesized in the hypothalamus and transported to the posterior pituitary gland from where it is released. Also called antidiuretic hormone.

Corticotropin-releasing hormone A hypothalamic peptide hormone that stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland.

Neurotrophic factor A family of proteins that induce the survival, development, and function of neurons.

Polyamines Compounds containing more than one amino group, such as spermine and spermidine.

Ribosomal RNA The RNA component of the ribosome essential for protein synthesis in all living organisms.

CHAPTER

Epigenetics in Posttraumatic Stress Disorder

16

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KEY CONCEPTS

- PTSD is not the inevitable conclusion of severe trauma; most trauma-exposed individuals will not develop the disorder.
- Low levels of cortisol and high HPA reactivity to stress characterize PTSD.
- Risk of PTSD is significantly associated with childhood abuse and trauma, although PTSD in those with a history of abuse may represent a biologically distinct subtype of the disorder.
- Risk of PTSD can be transmitted across generations.
- Stress and trauma have pronounced and persistent epigenetic effects on the brain and other tissues.

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ABBREVIATIONS

11β-HSD 11β-Hydroxysteroid dehydrogenase
ABN Arched-back nursing
ACC Anterior cingulate cortex
ACTH Adrenocorticotropin
AVP Arginine vasopressin
BDNF Brain-derived neurotrophic factor
CRH Corticotropin-releasing hormone
DNMT DNA methyltransferase
FGF2 Fibroblast growth factor 2
FKBP5 FK506 binding protein 5
GR Glucocorticoid receptor
H3K27 Histone H3 lysine 27
H3K9 Histone H3 lysine 9
HDAC Histone deacetylase
HPA Hypothalamic–pituitary–adrenal
LG Licking and grooming
LINE-1 Long interspersed nuclear element 1
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
MR Mineralocorticoid receptor
mRNA Messenger RNA
MS Maternal separation
ncRNA Non-coding RNA
NGFIA Nerve growth factor-inducible A transcription factor
PTSD Posttraumatic stress disorder
PVN Paraventricular nucleus of the hypothalamus
siRNA Small interfering RNA
SNP Single nucleotide polymorphism

Introduction

Anxiety disorders are among the most common forms of mental illness, with a lifetime prevalence in the U.S. population approaching 30%; they show a high degree of comorbidity with other mental disorders such as depression and alcohol abuse [1]. Posttraumatic stress disorder (PTSD), one of the most severe varieties of anxiety disorder, has a lifetime prevalence of roughly 7% in the United States (although levels are often higher in areas heavily affected by violence or disaster). The disorder shows differential incidence with regard to sex, as women show a higher prevalence than men [2]. PTSD usually occurs as a consequence of exposure to severe stressors such as combat, child abuse, or rape. Diagnostically, it is characterized by three symptom clusters: re-experiencing, avoidance, and hyperarousal [3]. Stress is a factor in the pathogenesis of most anxiety disorders, and this clear environmental influence in these disorders is a significant reason why they have attracted attention from researchers interested in epigenetic influences on human disease. PTSD was originally construed as the inevitable consequence of exposure to an overwhelming stress; however, in the decades since the identification of PTSD as a distinct disorder, it has become clear that, despite the fact that a majority of people will be exposed to a severe trauma at some point in their life, most will prove resilient to it and only a minority will develop PTSD [4].

Posttraumatic stress disorder is characterized neuroanatomically by changes in the volume and activity of stress-reactive brain regions, including the hippocampus, prefrontal cortex, and amygdala. Generally, the activity of the amygdala has been observed to increase while that of medial prefrontal structures is reduced. The hippocampus shows a decline in volume in PTSD but may be more metabolically active nonetheless [5]. Stress in both humans and animal models plays a significant role in sculpting all of these regions from the level of regional volumes down to the relative exuberance of dendritic arbors or the birth of new neurons [6,7].

Differential susceptibility to PTSD is clearly influenced by familial and early-life environment [8,9]. Early-life adversity has gained more attention as of late as evidence has mounted to support its role in PTSD susceptibility, as in a variety of other physical and mental disorders [10,11]. To give

one highly significant example, a recent study provides evidence that PTSD that arises in individuals with a history of child abuse may be biologically distinct from PTSD in non-abused adults [12]. Genetic approaches have certainly made progress in identifying gene polymorphisms that correlate with PTSD risk, but the contributions of these genetic factors is relatively small, and the problem of "missing heritability" remains [13]. Further, PTSD is one of the few mental disorders where transgenerational non-genetic factors clearly play a role (see below). For these reasons, epigenetic factors are likely to play a significant role in the etiology of the disease and have become an important focus of research.

The hypothalamic–pituitary–adrenal (HPA) stress axis represents another facet of the pathogenesis of PTSD. Patients with PTSD have long been known to show lower basal levels of corticosterone and aberrant HPA responses to stress (see below). Many, if not most, of the findings regarding epigenetic influences in PTSD pertain to altered activity of different parts of the HPA axis' suite of regulatory mechanisms.

Whether or not a particular individual will develop PTSD or anxiety as a consequence of trauma is determined by the interplay of a number of internal and exogenous factors. Within the individual, the genome, epigenome, and HPA axis interact with each other, as well as with environmental insults such as abuse and trauma. This interplay begins in early life and extends throughout the lifespan, determining susceptibility or resilience via changes in the structure and function of the epigenome and neuroendocrine axis (see Figure 16.1).

The lasting impact of early adversity

Early-life environment, from conception through adolescence, is manifestly important in deciding the successful function of a variety of systems in later life. Sensitive periods are well described for a number of capacities from mother–infant bonding to vision, and, as will be made plain below, the stress axis is no exception in this regard. Severe maternal stress during pregnancy, of the sort that occurred during the Dutch Hunger Winter of 1944–45, can have lasting effects on the resulting offspring. The children of starving mothers had higher risk for a number of psychiatric and medical disorders, as well as a hyperreactive HPA axis and epigenetic alterations in metabolic genes well into middle age [14,15]. Abuse and other forms of severe childhood adversity are clearly factors in PTSD vulnerability, as they are in a diverse array of mental and physical disorders ranging from heart disease to drug abuse [10], particularly those disorders in which stress plays a role [8]. The influence of early adversity might even be said to begin before conception in the case of PTSD, as it is one of a few human disorders where a clear pattern of epigenetic transgenerational inheritance occurs [9]. This appears to be true in both the broad sense of the term epigenetic, as well as the strictly molecular sense more commonly used. Transgenerational epigenetic inheritance has been hypothesized to occur in three possible ways: fetal programming effects, behavioral transfer, and germline transmission [16]. It is evident that all three of these mechanisms could play a role in PTSD and other stress-related disorders.

In rodent models, early stress, treatment with corticosteroids, or administration of the neurotrophic factor fibroblast growth factor 2 (FGF2) produces an accelerated development of fear learning. This appears to be accompanied by earlier myelination in both the whole brain and the amygdala [17]. Accelerated development of fear learning has an obvious relationship with PTSD and other stress and anxiety disorders, as PTSD is characterized by impaired capacity to extinguish fear learning [18]. White matter abnormalities and reduced regional volumes are also present in PTSD, particularly in the anterior

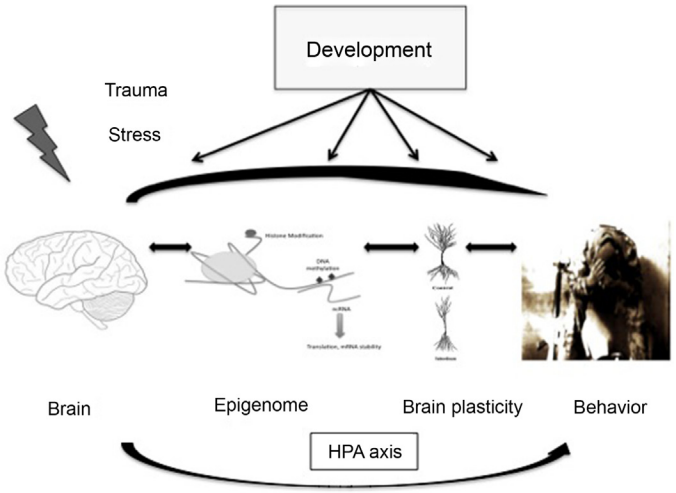

FIGURE 16.1

Susceptibility to PTSD is determined by the interplay of stress and trauma across development with the brain, epigenome, and HPA axis to produce the behavioral syndrome of PTSD or resilience.

Adapted from Hunter RG, McEwen BS. Stress and anxiety across the lifespan: structural plasticity and epigenetic regulation. Epigenomics 2013; 5: 177–94.

cingulate cortex (ACC) and hippocampus (regions vital to contextual fear conditioning and extinction learning), with the amygdala showing a more complex response to accumulated trauma. The cingulum, which connects the hippocampal region with the ACC, shows reduced integrity in PTSD patients relative to traumatized controls [19]. Reductions in ACC volume have been shown to occur largely in PTSD victims who suffered trauma prior to 13 years of age [20,21]. This distinction is one of several suggesting that PTSD consequent to trauma in early life may represent a biologically distinct disorder from PTSD acquired in the absence of childhood trauma or abuse. Perhaps the most compelling piece of evidence in this regard is a recent study by the Ressler and Binder laboratories which found vastly different patterns of gene expression and DNA methylation in the peripheral blood of patients with a history of childhood adversity than in patients who developed the disorder in the absence of a history of early-life trauma [12]. This finding is supported by other recent work examining the effect of parental stress on persistent patterns of DNA methylation in children and adolescents [22]. This approach is interesting as it examined the forest of expression patterns rather than the trees of individual gene polymorphisms, and as such may point the way toward a new approach to examining genetic and epigenetic influences in complex disease. These findings also suggest that it may be possible to develop a biological test for abuse history, which would also provide a means for distinguishing PTSD endophenotypes.

HPA axis in PTSD and stress-related disorders

The HPA axis has long been a focus for PTSD research because it is the central axis for the control of the stress response. The classical stress response originates in the paraventricular nucleus

of the hypothalamus (PVN). Parvocellular neurons from the PVN release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the pituitary portal circulation where they can activate cells in the anterior pituitary and cause the release of adrenocorticotropin (ACTH) into the general circulation. ACTH, in turn, induces the adrenal cortex to secrete cortisol (in primates) and/or corticosterone (the principal corticosteroid in rodents). These hormones then bind to their receptors—mineralocorticoid receptor (MR) and glucocorticoid receptor (GR)—throughout the body. The limbic structures of the brain are particularly rich in corticosteroid receptors, as are the anterior pituitary and PVN. MR, which has a higher affinity for corticosteroids, is thought to be important under basal conditions and during stress onset, while GR is thought to be involved in the termination of the stress response [23], most importantly by closing the negative feedback loop at the level of the hypothalamus.

Above the level of the hypothalamus, the central nucleus of the amygdala helps orchestrate the release of norepinephrine from neurons of the locus coeruleus into their terminal fields, comprising much of the forebrain, including the PVN. The PVN responds to this increased noradrenergic input by raising its own response, as evidenced by studies demonstrating blunted stress responses in animals where the sources of noradrenergic input into the PVN have been lesioned and increased activity when these areas are stimulated exogenously [24,25].

It is worth noting here that the acute activation of the stress axis is an adaptive (and adapted) response. Most mammals, humans included, are very resilient to brief stressors. Glucocorticoids improve memories of stressors, though they impair memory and attention to aspects of the environment unrelated to the source of stress [26,27]. Pathology is much more likely to arise when stress is chronic or inescapable [28].

In PTSD, the HPA axis shows consistent abnormalities. The most consistent of these is, paradoxically, low cortisol levels [29,30]. This finding does not appear to vary across culture or sociodemographic circumstance [31], although it is more pronounced in that population of PTSD patients with a history of abuse in early life [32]. The low levels of circulating cortisol in PTSD are not a result of low ACTH release or a general deficit in HPA responsiveness, as stress-evoked cortisol levels are normal to supranormal. The deficit, particularly in patients with a history of early-life trauma, appears to be, at least in part, due to increased sensitivity to corticosteroids in target tissues resulting from increased expression of GR [31].

Corticosteroid metabolism also differs from the norm in PTSD sufferers. The enzyme 11β-hydroxysteroid dehydrogenase 2 (11β-HSD2), which catalyzes the inactivation of corticosterone and cortisol, shows increased promoter methylation and reduced expression as a consequence of prenatal adversity in animal models [33]. Indeed, in humans, aberrations in maternal diet during gestation can produce changes in 11β-HSD2 promoter methylation and gene expression decades after birth. Holocaust survivors show persistent alterations in the ratio of urinary corticosteroid metabolites suggestive of abnormal levels of both 11β-HSD2 and 5α-reductase (another enzyme involved in corticosteroid catabolism, especially in the liver), with the greatest alterations seen in those who were youngest during the time of exposure [34]. Similarly, children of mothers who were pregnant and developed PTSD as a consequence of the 9/11 atrocity showed low salivary cortisol at one year of age, again pointing to the significance of developmental programming of the HPA axis in PTSD and other stress-related anxiety disorders. Significantly, 5α-reductase levels appear to be predictive of treatment response to psychotherapy for PTSD, as patients expressing lower levels of the enzyme were less likely to see an effect of treatment than those who showed normal expression of the enzyme [35].

The function of the HPA axis is also influenced by the function of the chaperone proteins, which interact with GR within the cell. Most notable of these in the context of PTSD is FK506-binding protein 5 (FKBP5), a protein that interacts with the GR chaperone complex in the cytosol to reduce both the GR's affinity for corticosteroids and its probability of translocation into the nucleus [36]. Thus, changes in FKBP5 function or expression would be predicted to alter the sensitivity of glucocorticoid feedback within the HPA axis. Indeed, single nucleotide polymorphisms (SNP) in the FKBP5 gene have been shown to associate with PTSD symptoms [37]. Most significantly, childhood trauma has been linked to allele-specific demethylation of the glucocorticoid response elements in the FKBP5 gene [38], and SNPs in the gene are associated with PTSD risk as a consequence of childhood trauma [39].

It has been hypothesized that the hypervigilant PTSD phenotype could be adaptive in adverse environments, and as such the disorder might be seen as the result of adaptive programming to the future environment predicted by adverse early-life events [31]. Fortunately for our society, but unfortunately for PSTD patients, this sort of response is inappropriate and maladapted to most life situations in the developed world. However, this hypothesis does point in the direction of early identification and intervention in cases of early-life trauma and abuse in order to reduce the risk of developing the full syndrome in later life.

Epigenetic effects of stress

Stress and trauma in development

It is well established that stressful events can have a lasting impact on the brain and behavior in both humans and animal models, so it was only logical that as the methods of molecular epigenetics became available that stress researchers would turn their attention to them. Stress, and stressful events such as fear conditioning, have been show to alter levels of a variety of epigenetic marks in the brain and other tissues [40,41]. These effects begin as early as gestation, appear to continue throughout the lifespan, and may be visited unto the third generation or beyond.

Stress can cause changes in behavior and brain physiology even during fetal development [42]. The lasting effects of prenatal stress can include altered HPA axis sensitivity, increased depressive and anxious behavior, as well as learning deficits which are mediated in part by the effects of stress and stress hormones on sensitive brain regions such as the prefrontal cortex, amygdala, and hippocampus [40,43]. With regard to epigenetic marks, it has been demonstrated that prenatal stress in rodents increases methylation of the stress-responsive GR 1–7 promoter while decreasing CRH promoter methylation. These changes correlate with altered DNA methyltransferase activity in the placenta [44]. More interestingly, some of these changes can persist across at least two generations and seem to be mediated in part by changes in miRNA activity [45]. Human infants exposed to maternal stress and depression *in utero* show similar changes in GR methylation [46]. Prenatal stress in animals can also alter the methylation and expression of 11β-HSD2, which metabolizes corticosteroids as well as DNA methyltransferase (DNMT) levels in the fetal cortex [47]. In humans, increased placental 11β-HSD2 promoter methylation is associated with slower infant growth and behavioral deficits [48], and, as mentioned above, 11β-HSD2 activity appears to be altered in PTSD patients, suggesting that this might be at least part of the means by which PTSD risk could be transmitted transgenerationally. The offspring of rat dams subjected to restraint stress during pregnancy show increased expression of DNMT1 and 3a, and decreased GAD67 and RELN expression in the

hippocampus and prefrontal cortex. The reduced expression in the latter two genes is likely due to increased DNA methylation of their promoters. These mice show a suite of behavioral abnormalities including reduced social interaction and impaired fear conditioning, suggestive of a vulnerable phenotype. These defects were reversed by treatment with the histone deacetylase (HDAC) inhibitor valproate or the atypical antipsychotic clozapine [49]. Neonatal handling can also reduce the long-term negative consequences of prenatal stress [50]. This is proof of principle, at least, that the deficits induced by prenatal stress are treatable and that early intervention in at-risk children may be able to increase their resilience to later shocks.

Stress and adverse environments during infancy and childhood also have marked epigenetic effects. The epigenetic impact of the early postnatal environment has been best described by Meaney and collaborators. This group established that natural variations in maternal care by rodent mothers predicted stress responsiveness and HPA activity in their adult offspring. Rat mothers show different levels of arched-back nursing (ABN) and licking and grooming (LG) behaviors toward their young, and those mothers with low LG–ABN levels produce adult offspring with lower levels of hippocampal GR and higher levels of CRH in the PVN than mothers who fall on the high end of the distribution for these behaviors [51–53]. Low LG–ABN offspring also show reduced dendritic arbors and spine numbers in the hippocampus, although they show superior learning in stressful circumstances [54,55]. The most significant fact about these observations is that both the HPA and the maternal behavioral phenotypes were transmitted across multiple generations, making this model perhaps the clearest demonstration of transgenerational epigenetic inheritance in the research literature on the subject.

Through collaboration with Moshe Szyf, the Meaney group has been able to describe the molecular mechanisms by which this inheritance occurs in great detail. The reduced hippocampal GR expression appears to be due to hypermethylation of the GR 1–7 promoter in adult low LG–ABN pups. This appears to be behaviorally transmitted, as cross-fostering of low LG–ABN pups to high LG–ABN mothers normalizes promoter methylation at the 1–7 locus. Higher levels of maternal care result in increased binding of NGFIA/Egr1 protein to the 1–7 promoter and consequently lower levels of histone acetylation in the region. Histone acetylation is generally inversely correlated with DNA methylation, so the HDAC inhibitor trichostatin A was used to switch the levels of these marks on the GR promoter and thereby rescue both GR receptor levels in the hippocampus and the stress-reactive phenotype in low LG–ABG animals [56,57].

In humans, the equivalent genomic locus of the 1–7 promoter in rodents is the 1F promoter [58], and, like the rodent promoter, it too shows differential methylation in response to early-life environment. It has been shown to be hypermethylated in the brains of suicide completers with a history of child abuse, and this differential methylation appears to extend to other regions of the GR promoter as well [59,60]. In normal adults with a history of abuse or adversity, GR hypermethylation has been observed in peripheral blood and associated with a lower cortisol response to dexamethasone challenge [61]. This is consistent with the enhanced negative feedback observed in PTSD as well as trauma-exposed individuals in general [62]. It should be noted that a number of recent studies have made clear that the epigenetic and transcriptional effects of early adversity in humans are not limited to GR; indeed, the effects of abuse may extend to thousands of loci throughout the genome [12,22,63]. Much remains to be learned in this area with regard to both PTSD risk and the syndrome resulting from early-life adversity of which PTSD might be regarded as but one expression.

Other early-life insults have also been shown to produce transgenerational effects on the brain and behavior. Treatment with synthetic glucocorticoids during fetal development has been shown to

alter DNA methylation levels in both the fetus and placenta, in addition to altering the expression of the DNA methyltransferase DNMT3b [64]. Not all transgenerational epigenetic transmission is maternal; some appears to come from the father. Skinner and Crews and various colleagues have shown that vinclozolin, a fungicide with antiandrogenic properties, produces paternally transmitted effects on anxiety and stress responsiveness that persist across generations [65,66] and are mediated by direct effects on the sperm epigenome [67]. Dietz et al. also reported paternal transmission of stress-induced behavior. Offspring of socially defeated male mice show a higher susceptibility to defeat themselves in adulthood [68]. Although it is not yet established, it is quite possible that similar mechanisms could exist in humans and provide an explanation for some of the missing heritability in psychiatric genetics.

Other models of early-life adversity also show evidence of epigenetic effects on stress-related behavior. Maternal separation (MS), a paradigm where rodent pups are separated from their mothers for extended periods during the first days of life, also shows transgenerational effects on stress reactivity which have a differential effect on males compared with females and may be paternally transmitted [69]. MS produces increased HPA reactivity to stress, increased anxiety in response to stress, and alterations in hippocampal plasticity and learning in adulthood [6,70]. This phenotype results in part from increased ACTH release in response to stress resulting from increased CRH and AVP secretion from the PVN [71]. Murgatroyd et al. [72] demonstrated that MS increased expression of AVP, though not CRH, and that this in turn was due to hypomethylation of a methyl-CpG-binding protein 2 (MeCP2) binding site in the AVP promoter. MeCP2 is implicated in a number of neurological disorders, as well as in the regulation of stress and plasticity genes such as brain-derived neurotrophic factor (BDNF) [73]. Stressed and abusive dams provide another rodent model of early-life adversity, and here, too, epigenetic effects have been observed, specifically via hypermethylation of the BDNF promoter [74]. Alterations in DNA methylation and DNMT activity play a critical role in the development of trait anxiety [75] and stress responsiveness in this critical time period, establishing that the early environment is a sensitive period for the epigenetic modulation of the traits that contribute to adult vulnerability to anxiety and stress-related disorders.

Adolescence is a time period marked by the shift from the rapid growth and parental orientation of childhood toward reproductive maturity and an orientation toward the wider social world. It is marked by a number of changes in the structure and function of the brain and HPA axis [76,77]. It is also a vulnerable period for a variety of psychiatric disorders [78]. Research into the epigenetic impact of stress during this developmental stage has been relatively scant, however. This has begun to change recently, most notably with a study demonstrating that mild stress in adolescent mice causes a GR-dependent hypermethylation of the tyrosine hydroxylase promoter and changes in the mesolimbic dopamine system which lead to a number of behavioral deficits as well as altered HPA reactivity [79]. It is to be hoped that further work is done regarding this critical time period in brain development and disease susceptibility.

Stress and trauma in adult life

Stress during adulthood is often the trigger that changes a pre-existing vulnerability into a clinical case of PTSD. Clearly, stress during adult life can have marked effects upon the brain and behavior. Brain regions such as the amygdala, prefrontal cortex, and hippocampus are highly susceptible to plastic changes in response to stress [6]. The hippocampus in particular not only is highly stress susceptible but also expresses a large variety of epigenetic reader and writer genes [40]. This makes

it a particularly interesting region with regard to the present topic, and for this reason much work has been focused on the hippocampus, although the region is certainly not the only one involved in PTSD or anxiety, nor is it the only site in the brain where epigenetic changes are observed.

Fear conditioning is an acutely stressful model with relevance to PTSD [80] and was one of the first systems in which molecular epigenetic influences on behavior were examined. One of the earliest observations was that fear conditioning altered hippocampal histone acetylation. Subsequent work established that fear conditioning also modulated DNMT expression and DNA methylation at a number of genes in the hippocampus, and that modulation of both histone acetylation and DNA methylation are necessary for proper fear learning [80]. The Schafe laboratory has recently established that DNMT activity is required for proper fear conditioning and neural plasticity in the amygdala [81].

Histone deacetylases figure prominently in the mechanism by which positive manipulations such as environmental enrichment are able to increase synaptic plasticity and protect against the potentially maladaptive structural effects of stress. The Tsai lab [82,83] has demonstrated that the positive effects of enrichment upon the anterior cingulate are mediated by the neuron-specific HDAC2, which modulates a relatively select group of genes, notably glutamate receptor subunits and BDNF, an effect mediated in part by GR. Indeed, blockade of HDAC activity blocks the ability of BDNF to promote spinogenesis and excitatory neurotransmitter release in the hippocampus [84].

One of the earliest findings explicitly linking stress with neuroepigenetic marks was that by the Reul group [85], who showed that an acute stressor, forced swimming, increased the levels of the dual phospho-acetyl mark, H3S10phos/K14ac, on histone H3 within the rat dentate gyrus. This mark, which is associated with transcriptional activation, was one of the first identified as being subject to exogenous manipulation in the brain with a number of neurotransmitter agonists [86]. The increase in H3S10phos/K14ac is also observed in exercise and as such may be a marker for an adaptive rather than maladaptive response to stress. The mark increases in response to combined corticosteroid and glutamatergic stimulation and declines in response to the activation of GABAergic inputs. The induction of this mark by combined corticosteroid and excitatory amino acid transmission is typical of stress-induced changes in the hippocampus; however, induction of the mark seems necessary for proper memory of the stressful event [87], which suggests it might be a useful target for acute pharmacotherapy of trauma.

Social defeat stress in rodents uses agonistic encounters between two individuals to produce a syndrome analogous to human depression, though with potential implications for PTSD as well [88]. The Nestler group [89,90] has used this model to examine the epigenetic underpinnings of psychiatric disease, antidepressant action, and resilience. They found that defeat is associated with increases in the repressive histone H3 lysine 27 trimethyl mark (H3K27me3) in the BDNF promoter within the nucleus accumbens. The antidepressant imipramine, which rescues the behavioral deficits seen in defeat, also reduces repressive histone marks and increases activating marks such as histone H4 acetylation and H3K4 trimethylation. Further study revealed that these changes were linked to changes in HDAC activity, and HDAC5 was found to mimic the properties of antidepressants in protecting against the behavioral effects of chronic stress or cocaine [91]. DNA methylation in the accumbens was also linked to chronic social defeat, as DNMT3a expression increased in defeated animals, and the increase was found to be correlated with plastic changes in dendritic spines [92]. Much like humans, a substantial fraction of mice show resilience to defeat stress; in these animals, DNA methylation of the CRH promoter in the PVN was maintained while in defeated animals it was demethylated [93]. In the accumbens, resilience and antidepressant action correlate with the ability to

maintain normal levels of histone H3K9 and H3K27 methylation in response to defeat [94,95]. In the hippocampus, complex changes in histone methylation are also observed as a result of both chronic and acute restraint stress. In chronic stress, global levels of the active transcriptional mark H4K4me3 are moderately decreased. In acute stress, substantial rapid changes in levels of both H3K27me3 and H3K9me3 are observed [96]. These studies suggest that increased H3K9 methylation and HDAC inhibition both promote resilience to stressful events.

Of course, DNA methylation and histone modifications are not the only forms that molecular epigenetic mechanisms take. Non-coding RNAs (ncRNA) come in a variety of different flavors and functions, and they too have been implicated in the interactions of stressors with the nervous system. Several microRNAs (miRNAs), notably miR-124a and miR18a, have been implicated in the regulation of GR levels in the brain [97,98]. Other miRNAs are induced in the brain by stress, often in a regionally specific fashion [99]. Stress increases miR-34 in the amygdala, which in turn reduces expression of the CRHR1 receptor to produce an anxiolytic effect on behavior. Interestingly, mice lacking Dicer, a necessary constituent of the miRNA processing machinery, showed increased anxiety [100]. miRNAs may also play a role in the paternal transmission of stress memory across generations, as the Bale laboratory [101] has recently shown that paternal stress alters sperm miRNA content and the HPA axis of the offspring of these fathers.

Genes and miRNAs constitute a relatively small fraction of the genome, typically around 5%; much of the rest in humans, as in most multicellular organisms, is composed of transposable elements [102]. These elements were long thought to be silent genomic "junk" [103], but more recent work by the Encyclopedia of DNA Elements and other projects has shown that these elements are transcriptionally active in a tissue- and cell-type-specific fashion and that they have some measure of control over both their own transcription and that of adjacent genes [104–106]. This represents a vindication of their discoverer, Barbara McClintock, choosing to describe them as "controlling elements" [107,108]. These elements are perhaps more active in the brain than in many other tissues and are certainly capable of transposition in the adult hippocampus as well as in a variety of brain areas during development. More recently they have been implicated in a number of neurodegenerative disorders in both humans and model organisms [102,109]. Significant to the study of PTSD, some of these elements are subject to epigenetic regulation by stress in both the hippocampus and the amygdala of rodents [110,111]. In peripheral blood from combat-deployed veterans, hypermethylation of long interspersed nuclear element 1 (LINE-1) transposable elements prior to deployment was found to correlate with not developing PTSD; hypomethylation of these elements after deployment correlated with developing the disorder. Another class of transposons, the Alu elements, was also found to show a pattern of reduced methylation during the course of deployment only in those soldiers who developed PTSD [112]. These findings make clear that it is likely that PTSD risk, and potentially the pathogenesis of the disorder, is at least partly under the control of this genomic dark matter. Indeed, transposable elements could do much to explain missing heritability in PTSD, as well as a number of other neuropsychiatric disorders.

Implications of epigenetics for the treatment of PTSD and related disorders

At present, no novel pharmacotherapies for PTSD have been developed with the goal of targeting the epigenetic machinery. However, it has emerged that many drugs with lengthy clinical pedigrees do, in fact, act upon epigenetic targets [13]. Fluoxetine has been shown to alter levels of miR-16, a miRNA that modulates serotonin transporter expression [113]. Amitriptyline, which is used to treat many of

the symptoms of PTSD, acts directly on the histone methyltransferase G9a and indirectly upon DNA methylation via DNMT1 [114]. Imipramine is a HDAC inhibitor, and other psychiatric drugs such as haloperidol, clozapine, paroxetine, and citalopram have been shown to have activity against both DNA and histone-modifying enzymes [13,114]. The extent to which these actions are separable from the long-described actions of these drugs upon neurotransmitter receptors is largely unknown at present and awaits further study.

It is also clear that the identification and treatment of children with exposures to adverse circumstances will be a significant factor in reducing the incidence of PTSD in the population. Whereas these interventions are inherently more complex and difficult than drug treatment, the potential of preventing not only a substantial fraction of PTSD cases but a multitude of other medical and psychiatric disorders as well argues strongly for such efforts. Progressive, controllable exposures to manageable stressors could go a long way toward developing the sort of "quotidian resilience" that likely underlies the observation that most trauma-exposed individuals do not develop lasting psychiatric distress as a consequence of trauma [115,116], although in this area, too, much research remains to be done.

Conclusions

It would be an understatement to say that epigenetic factors play a role in PTSD risk and pathogenesis. These factors have effects that are clearly transmitted across generations via mechanisms spanning the molecular to the behavioral. This is as true in human patients and their families as in animal models of the disorder. Although this realization represents a profound advance in our understanding of the disease, much remains to be understood.

Perhaps the most significant problem for psychiatric epigenetics in PTSD, as in other disorders, is how to translate the knowledge we have acquired in the past decade or so into clinically useful techniques and guidance. Psychiatric drugs (and for that matter cancer drugs), many decades old, act on epigenetic players; however, this new understanding of old drugs has yet to improve upon these manifestly imperfect drugs. Much work has been done to develop selective inhibitors of the numerous identified enzymes involved in regulating the epigenome, yet it is unclear if these will substantially improve upon the efficacy and side-effect profile of existing drugs. Off-target effects are at least as likely with drug targets as ubiquitous as DNMTs or HDACs as they are with less numerous and more selectively distributed neurotransmitter receptors.

Important questions about how trauma and adverse environments interact with the brain and the epigenome remain. How is child abuse transduced into the forest of epigenetic and transcriptional changes observed years or decades after abuse has ceased? How do these changes lead not only to increased risk of PTSD and anxiety disorders but also to risk for so many other disorders as well? How do we defuse the propensity to PTSD that is handed down through the generations? No doubt, rapid technical advances in next-generation sequencing, cell-specific manipulations of neurons such as optogenetics, etc., will play a role, so much deeper research into the social transmission of behavior and the endocrine and physiologic correlates of trauma and resilience is necessary.

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Glossary

Corticosteroid Any one of a number of steroid hormones secreted by the adrenal cortex. In humans, the major corticosteroid is cortisol; in rodents, corticosterone. Mineralocorticoids, such as aldosterone, regulate blood volume and renal function. Glucocorticoids, such as cortisol, regulate immune function and glucose metabolism but are most significantly associated with the stress response.

HPA axis The neuroendocrine stress axis, comprised of the PVN of the hypothalamus, anterior pituitary corticotrophs, and adrenal cortex.

Non-coding RNA (ncRNA) Any of a number of RNAs that do not code for a protein. These include small interfering RNA (siRNA) and microRNA (miRNA), which are actively involved in the regulation of messenger RNA (mRNA) and protein expression, as well as a growing number of more exotic species.

Neurotrophic Having a trophic effect on neurons, including changes in spinogenesis, dendritic architecture, axonal growth, and neurogenesis. A number of neurotrophic factors have been implicated in psychiatric disease, including brain-derived neurotrophic factor (BNDF) and fibroblast growth factor 2 (FGF2).

Spinogenesis The production of new dendritic spines, a common form of neural plasticity.

Transgenerational epigenetic transmission Transmission of acquired behavioral responses/susceptibilities across generations without any change in the coding sequence of genes/DNA.

Transposon A common element in mammalian (and other) genomes that is capable of transposing itself (moving) around the genome. Recent research has established that transposons are not transcriptionally silent as once thought, but are actively transcribed and may play a role in both normal physiology and disease. These include the human LINE-1 and Alu elements.

CHAPTER

Epigenetics and Cognitive Disorders

17

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KEY CONCEPTS

- Epigenetic processes, including DNA methylation and histone tail modifications, play a fundamental role in the neuronal plasticity required for learning and memory.
- Global changes of the epigenome occur either in familial or sporadic forms of neurodegeneration and might be responsible for several of the cognitive and motor symptoms accompanying those disorders.
- Studies in animal models of neurodegenerative diseases showed that epigenetic drugs are able to restore both cognitive and motor functions, thereby opening a window for potential therapeutic interventions.

ABBREVIATIONS

5-aza-dC 5-Aza-2′-deoxycytidine
5-CH_3-THF 5-Methyltetrahydrofolate
5-hmC 5-Hydroxymethylcytosine
5-LOX 5-Lipoxygenase
5-mC 5-Methylcytosine
Aβ Amyloid-beta peptide
AD Alzheimer's disease
APP Amyloid precursor protein
ATRX Alpha thalassemia X-linked mental retardation
BACE1 β-site APP cleaving enzyme 1
BD Bipolar disorder
BDNF Brain-derived neurotrophic factor
CBP CREB-binding protein
CNS Central nervous system
COX2 Cyclooxygenase 2 gene
CpG Cytosine–phosphate–guanine
CYP2E1 Cytochrome P450 2E1
DLB Dementia with Lewy bodies
DNMTs DNA methyltransferases
EED/WAIT1 Embryonic ectoderm development polycomb histone-methylation modulator
FAAH Fatty acid amide hydrolase
GPNMB Transmembrane glycoprotein NMB gene
H3K36 Histone H3 lysine 36
H3K4 Histone H3 lysine 4
H3K79 Histone H3 lysine 79
H3S10 Histone H3 serine residue 10
HATs Histone acetyltransferases
HCY Homocysteine
HD Huntington's disease
HDAC Histone deacetylase
HDACi Histone deacetylase inhibitor
HDMT Histone demethylase
HMT Histone methyltransferase
HTT Huntingtin
LBs Lewy bodies
LEARn Latent early-life associated regulation
LRRK2 Leucine-rich repeat kinase 2
MAT2A Methionine adenosyltransferase II alpha
MBD Methyl-CpG-binding domain
MCI Mild cognitive impairment
MeCP2 Methyl-CpG-binding protein 2
MET Methionine adenosyltransferase
MPP+ 1-Methyl-4-phenylpyridinium
MTR Methionine synthase
NEP Neprilysin
NFT Neurofibrillary tangle
PARK2 Parkin
PBMC Peripheral blood mononuclear cell
PD Parkinson's disease
PINK1 PTEN-induced putative kinase 1
PK Protein kinase
PP Phosphatase
PRMTs Protein arginine methyltransferases
PSEN1 Presenilin 1 gene
PSEN2 Presenilin 2 gene
SAH *S*-adenosylhomocysteine
SAHH SAH hydroxylase
SAM *S*-adenosylmethionine
SNCA alpha-Synuclein

SP Senile plaque
STX1B Syntaxin 1B gene
TET Ten–eleven translocation
THF Tetrahydrofolate
TMEM59 Transmembrane protein 59 gene
uH2A Monoubiquityl histone H2A

Introduction: role of epigenetics in memory and neurodegeneration

Epigenetic processes, such as DNA methylation, chromatin modifications, and non-coding RNAs, influence gene expression levels without involving changes of the primary DNA sequence, and they play a pivotal role in embryonic development, cell differentiation, and maintenance of cellular identity [1]. Epigenetic marks, such as DNA methylation, have been traditionally viewed as highly stable in postmitotic cells. However, postnatal brains appear to exhibit stimulus-induced methylation changes, at least in a few identified CpG dinucleotides [2], and there is consensus indicating that DNA methylation and histone modifications are dynamically regulated in the adult central nervous system (CNS) in response to experience [3]. The hypothesis that epigenetic mechanisms could regulate learning and memory was motivated by the need for stable molecular processes that evade the rapid turnover of the neuronal proteome [3]. Since then, increasing evidence supports a role for neural epigenetic modifications in the formation, consolidation, and storage of memory [3–5], and the processes that directly modulate chromatin structure to regulate gene expression are currently viewed as mediators of neuronal plasticity and long-term changes in behavior [4,5]. Indeed, the dynamic reversibility of epigenetic marks in neurons suggests that the epigenome can be quickly altered in response to experiential stimuli to regulate the expression of memory-related genes, including those necessary for the synaptic plasticity accompanying active learning. Certain of those regulatory changes stably persist throughout the lifespan, allowing stored memories to remain unchanged and dormant for extended periods of time until the conscious brain demands their recall [4,5]. Unfortunately, an increasing number of human diseases have at least part of their basis in aberrations of epigenetic processes and, given the pivotal role of epigenetics in memory formation, it is not surprising that changes of epigenetic marks might be at least partially involved in the onset and progression of cognitive disorders [6].

This chapter describes the changes in DNA methylation and histone modifications observed in cell culture models, animal models, and human tissues of three major neurodegenerative diseases: Alzheimer's disease (AD), Huntington's disease (HD), and Parkinson's disease (PD). Before addressing those points, the author provides a brief description, essential to understanding the next sections of this chapter, of epigenetic marks, their functional significance, and some of the most important writer, reader, and eraser proteins of the epigenome. A more detailed molecular description of epigenetic mechanisms and their role in physiology and pathology is provided in Chapter 2 of this book, and Chapters 4 and 5 detail the role of epigenetics in functioning of brain and neurons, as well as in learning, memory, cognition, neuronal plasticity, and neurotransmission.

DNA methylation

DNA methylation typically occurs in CpG islands (regions unusually enriched in the CpG dinucleotide) and represents one of the most important epigenetic mechanisms for gene regulation. It consists of

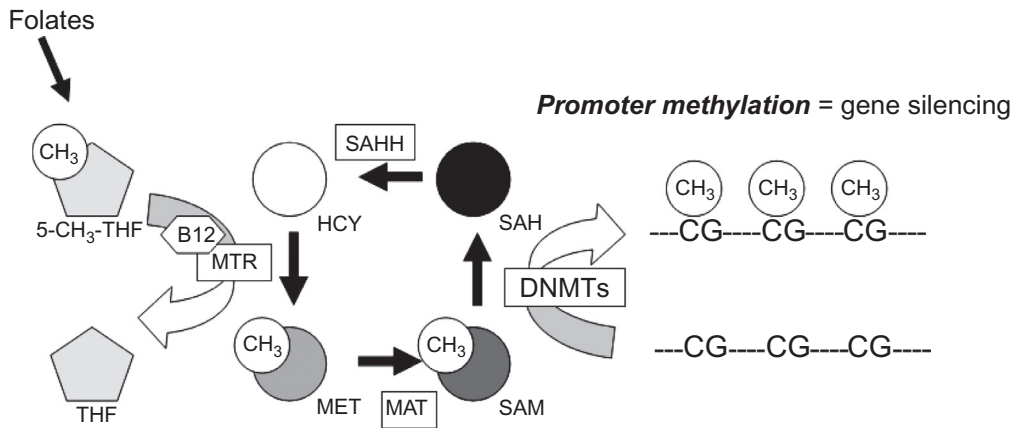

FIGURE 17.1

Schematic representation of the transfer of the methyl group (CH_3) from folate derivatives to DNA. Methionine synthase (MTR) transfers the methyl group from 5-methyltetrahydrofolate (5-CH_3-THF) to homocysteine (HCY), forming tetrahydrofolate (THF) and methionine (MET). Vitamin B_{12} (Vit B12) is a cofactor in this reaction. Methionine adenosyltransferase (MAT) uses MET to form *S*-adenosylmethionine (SAM), and SAM is then converted to *S*-adenosylhomocysteine (SAH) by DNA methyltransferases (DNMTs) that transfer the methyl group to DNA, leading to DNA methylation. SAH hydroxylase (SAHH) converts SAH to HCY, which can re-enter the DNA methylation cycle.

the addition of a methyl group (CH_3) to the 5′ position of the cytosine pyrimidine ring, forming 5-methylcytosine (5-mC). The reaction is mediated by the enzymes DNA methyltransferases (DNMTs) using *S*-adenosylmethione (SAM) as the methyl donor compound (Figure 17.1). Methylation of CpG islands in the promoter region of a gene might induce chromatin conformational modifications and inhibit the access of the transcriptional machinery, thus altering gene expression levels. Indeed, promoter methylation is commonly associated with gene silencing and promoter demethylation with gene expression [7].

Folate, other B group vitamins (B_2, B_6, and B_{12}), and homocysteine (HCY), participate in one-carbon metabolism, a complex metabolic pathway required for the production of SAM, the methyl donor compound in the reaction catalyzed by DNMTs (Figure 17.1). There are multiple families of DNMTs in mammals. DNMT1 is primarily involved in the maintenance of DNA methylation patterns during development and cell division, whereas DNMT3A and DNMT3B are the *de novo* methyltransferases and establish DNA methylation patterns during early development [8]. Methylated DNA is specifically recognized by a set of reader proteins called methyl-CpG-binding domain (MBD) proteins, such as MeCP2. MeCP2, MBD1, and MBD2 all contain a transcription repression domain to interact with other proteins and enhance DNA methylation-mediated transcriptional repression [9].

Enzymes of the ten–eleven translocation (TET) family convert 5-methylcytosine to 5-hydroxymethylcytosine (5–hmc), a modified form of cytosine hydroxymethylated at the 5′ position, which has lower affinity for MBDs than 5-mC [10]. The enzyme 5-methylcytosine hydroxylases TET by converting 5-methylcytosine to 5-hydroxymethylcytosine and promotes DNA demethylation

in mammalian cells through a process that requires the DNA base excision repair pathway [10]. 5-hmc is present at high levels in the brain and may not exclusively be an intermediate of an active demethylation process but rather an important epigenetic mark whose role in the brain is detailed in Chapter 11 of this book. Observations from mutants in reader proteins of the DNA methylation mark, including mice lacking *MECP2* or *MBD1* genes and Rett syndrome patients (a human disease caused by *MECP2* mutations), provided early evidence for a role of DNA methylation in memory, supported by subsequent evidence from pharmacological and genetic manipulations of DNMTs, overall implicating DNA methylation in adult memory consolidation [3].

Histone modifications

The chromatin state represents another important modulator of gene expression profiles. The nucleosome represents the structural and functional unit of chromatin and consists of approximately 147 DNA base pairs wrapped twice around a histone octamer consisting of two copies each of the core histones H2A, H2B, H3, and H4. Nucleosomes are connected by stretches of "linker DNA" and linker histones, such as histone H1, and are involved in chromatin compaction [11]. Chromatin can exist in a condensate inactive state (heterochromatin) or in a non-condensate and transcriptionally active state (euchromatin). Histone modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, and other posttranslational modifications [12]. The combination of these modifications and their interplay with DNA methylation and chromatin remodeling proteins regulate the chromatin structure in a dynamic fashion [12].

Acetylation occurs at specific lysine residues on the amino-terminal tails of the histones, neutralizing their positive charge and decreasing their affinity for DNA. As a consequence, histone acetylation alters nucleosomal conformation, which can increase the accessibility of transcriptional regulatory proteins to chromatin templates. Histone acetyltransferases (HATs) catalyze the acetylation of lysine residues in histones, whereas histone deacetylation is mediated by histone deacetylases (HDACs) [12]. Methylation on either lysine or arginine residues of histone tails can be associated with either condensation or relaxation of the chromatin structure, as several sites for methylation are present on each tail thus allowing different combinations [13]. For example, methylation of H3K4, H3K36, and H3K79 and histone acetylation are generally linked to an open chromatin structure that allows gene transcription, whereas histone hypoacetylation and increased di- and trimethylation at H3K9, H3K27, and H4K20 are considered repressive marks (Figure 17.2). Histone lysine methyltransferases (HMTs) and protein arginine methyltransferases (PRMTs) are the two classes of proteins that transfer the methyl group from SAM to histone tail residues, and histone demethylases (HDMTs) are the "erasers" of that mark [13]. Several other histone-modifying enzymes, including protein kinases, phosphatases, and ubiquitilases, are involved in the establishment of complex patterns of posttranslational modifications of histones (the so-called histone code) that tightly regulate gene expression levels (Figure 17.2). For example, phosphorylation of H3 at serine residue 10 (H3S10), enhances H3K4 methylation and H3K14 acetylation and is linked to gene transcription [12].

Numerous studies in invertebrates and mammals have shown that the activity of epigenetic enzymes including DNMTs, HATs, HDACs, HMTs, and HDMTs is required for learning and memory [4,5]. As a result, histone modifications and DNA methylation changes are observed in the hippocampus, amygdala, and cortex in response to learning, corresponding with the expression of memory-related genes and transcription factors [4,5], and there is increasing evidence suggesting that

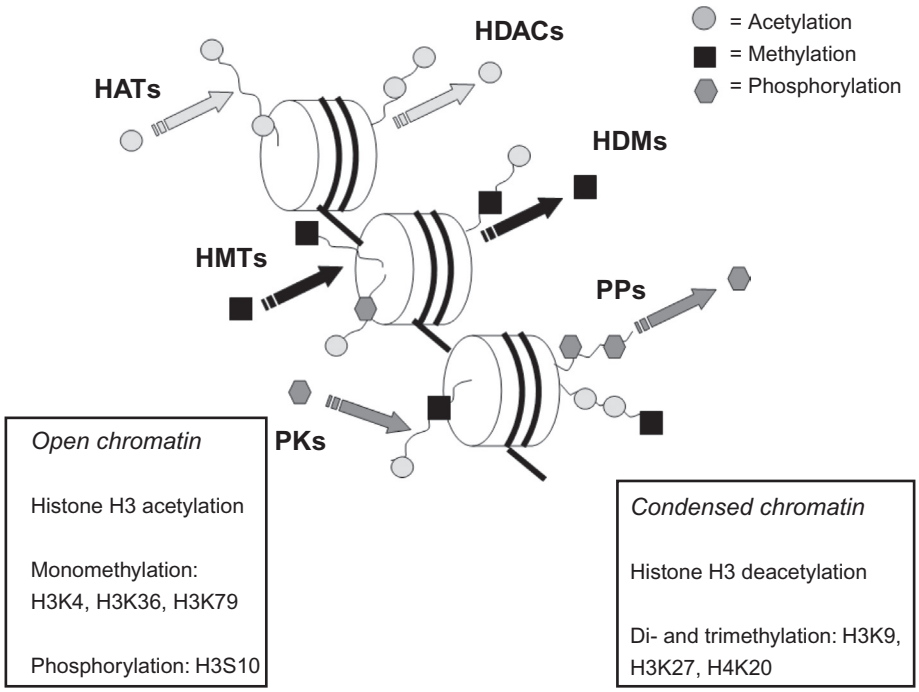

FIGURE 17.2

Some examples of histone modifications. Acetylation of lysine residues is mediated by histone acetyltransferases (HATs), and this mark is removed by histone deacetylases (HDACs). Histone methyltransferases (HMTs) and demethylases (HDMTs) are the writers and erasers of histone methylation; phosphorylation of serine residues depends on the action of protein kinases (PKs) and phosphatases (PPs). Some examples of marks associated with condensed chromatin and transcriptional repression, or with relaxed chromatin and transcriptional activation, are provided in the boxes.

manipulation of histone acetylation patterns through HDAC inhibition may represent a promising therapeutic approach to counteract cognitive decline in neurodegenerative diseases [6]. This last point is discussed in detail in Chapter 27 of this book.

DNA methylation changes and histone modifications in Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disorder and the primary form of dementia in the elderly, clinically characterized by a progressive neurodegeneration in selected brain regions, including the temporal and parietal lobes and restricted regions within the frontal cortex and the cingulate gyrus. The term dementia describes a set of symptoms including loss of memory, mood changes, and problems with communication and reasoning. Indeed, AD leads to memory loss

accompanied by changes of behavior and personality severe enough to affect daily life. The disease symptoms get worse over time, and available treatments may only help keep patients from getting worse for a limited time [6]. Affected brain regions in AD are characterized by the occurrence of extracellular amyloid deposits called senile plaques (SPs) and by the presence of neurofibrillary tangles (NFTs) composed of intra-neuronal aggregates of hyperphosphorylated tau protein. The primary component of SP is the amyloid β (Aβ) peptide, resulting from the proteolytic cleavage of its precursor, the amyloid precursor protein (APP), mediated by the protein secretases β-secretase (β-site APP cleaving enzyme 1, BACE1) and γ-secretase (a protein complex composed of presenilins and other proteins). Mutations in *APP* and presenilin (*PSEN1* and *PSEN2*) genes alter APP production and/or processing and cause early-onset (<65 years) familial forms of the disease, overall accounting for less than 1% of the total AD cases. However, most incidents of AD (90–95%) are sporadic forms diagnosed in people over 65 years of age, likely resulting from complex interactions among genetic, epigenetic, environmental, and stochastic factors superimposed on the physiological decline of cognitive functions with age [6].

Given the inaccessibility of human brain samples until postmortem and the paucity of available well-stored postmortem specimens, the contribution of epigenetic changes to AD and other neurodegenerative disorders has been largely investigated by means of disease models, such as neuronal cell cultures and transgenic animal models. However, studies performed on postmortem human brain specimens are increasingly available in the literature, and researchers are currently investigating whether peripheral blood cells of affected individuals can be informative of epigenetic changes. A summary of those studies is provided in Table 17.1.

DNA methylation changes in Alzheimer's disease: cell cultures and animal models

One of the most interesting lines of research in AD epigenetics takes as a starting point the hypothesis that impairments in folate metabolism, required for the production of SAM, could result in epigenetic changes relevant to AD pathogenesis [46]. The hypothesis came from observations of altered folate metabolism and SAM production in AD individuals [46]. Another field of active research in rodents and primates aims at investigating the possible epigenetic effects of early-life exposure to neurotoxicants [29,30]. Additional studies on epigenetic modifications of AD-related genes have been performed in cell cultures and disease animal models under conditions of oxidative stress or other stressors leading to increased Aβ peptide production [19,20,31,47,48].

B-vitamin restriction

Human neuroblastoma cells maintained under conditions of folate and vitamin B_{12} deprivation showed site-specific demethylation in the promoter of *PSEN1*, with subsequent increased production of presenilin1, BACE1, and APP proteins [14]. Similarly, B-vitamin (folate, vitamin B_6, and vitamin B_{12}) deficiency resulted in hyperhomocysteinemia, depletion of brain SAM, hypomethylation of specific CpG moieties in the *PSEN1* promoter, and enhancement of presenilin1 and BACE1 expression and Aβ deposition in a murine model of AD [15]. Particularly, it was shown that B-vitamin deficiency induces downregulation of *de novo* DNMT activities and increases demethylation activities in both neuroblastoma cell cultures and mice brain regions, leading to changes in the expression of proteins mainly involved in neuronal plasticity and mitochondrial function [16–18]. The same group observed that SAM supplementation induces an opposite tendency, restores *PSEN1* methylation levels, and reduces the progression of the AD-like features induced by B-vitamin deficiency in mice [14–17].

Table 17.1 Some Examples of DNA Methylation Changes in Alzheimer's Disease and AD Model Systems

Experimental Model	Observation	References
Human neuroblastoma cells and rodents	B-vitamins deprivation induced changes in *de novo* DNMT activities, demethylation in the promoter of *PSEN1*, increased production of the Aβ peptide, and changes in the expression of proteins involved in neuronal plasticity.	Fuso et al. [14–17], Borro et al. [18]
	SAM supplementation reversed the epigenetic effects induced by B-vitamin deprivation.	Fuso et al. [14–17]
Human and murine neuronal cell cultures	Exposure to Aβ oligomers induced global DNA hypomethylation.	Chen et al. [19], Shin et al. [20], Fuso et al. [14–17], Borro et al. [18], Maloney et al. [21], Bihaqi et al. [22,23], Dosunmu et al. [24], Bihaqi and Zawia [25,26], Li et al. [27], Hodgson et al. [28]
Rodents and monkeys	Early-life exposure to lead (Pb) resulted in inhibition of DNMTs, hypomethylation of the promoter of *APP* and delayed *APP* overexpression later in life, hypermethylation and repression of several other genes, and cognitive impairment in adulthood.	Basha et al. [29], Wu et al. [30], Bihaqi et al. [22,23], Dosunmu et al. [24], Bihaqi and Zawia [25]
Rodents	Early-life stress (maternal separation) resulted in promoter demethylation of *BACE1* and increased Aβ production.	Martisova et al. [31]
Postmortem AD human brains	Global reduction of 5-mC and 5-hmC contents	Mastroeni et al. [32,33] Chouliaras et al. [34]
	Changes in the methylation pattern of certain genes, including *TMEM59*, *SORBS3*, *S100A2*, *rDNA*, *COX2*, *BDNF*, and *CREB*	Bakulski et al. [35], Rao et al. [36] Siegmund et al. [37], Pietrzak et al. [38]
	No changes in the methylation of *APP*, *PSEN1*, *MAPT*, and *APOE* genes	Barrachina and Ferrer [39], Wan et al. [40]
Human AD blood cells	Increased levels of *LINE1* methylation	Bollati et al. [41]
	Increased methylation of subtelomeric regions of the shortest telomeres	Guan [42]
	Reduced methylation of certain genes: *FAAH*, *5-LOX*, and *PIN1*	D'Addario et al. [43], Di Francesco et al. [44], Arosio et al. [45]

Early-life exposure to lead

It was observed that developmental exposure of rats to lead (Pb) resulted in a delayed overexpression (20 months later) of the amyloid precursor protein and its amyloidogenic Aβ product [29]. Aged monkeys exposed to lead as infants responded in the same way [30]. Those observations led

researchers to formulate the "LEARn" (latent early-life associated regulation) model, suggesting that early-life epigenetic changes, induced by environmental agents, become latent and present themselves again at maturity or senescence to increase production of Aβ that may cause AD [21]. To further address this issue, the same group showed that developmental Pb exposure was accompanied later in life, in 23-year-old primate brains, by a significant decrease in the levels of proteins involved in epigenetic regulation, such as DNMTs, MeCP2, and proteins involved in histone modifications [22]. The analysis of global gene expression and methylation patterns in adolescent mice exposed to Pb as infants revealed that early-life exposure to the metal resulted in a repressed expression of genes related to the immune response, metal-binding, metabolism, and transcription/transduction coupling, and the repression of those genes corresponded with their DNA methylation profile [24]. Cognitive function in old age and its impact on biomarkers associated with AD were analyzed in rodents exposed to Pb during different lifespan periods [23]. That study revealed that cognitive impairment was observed in mice exposed as infants but not as adults, and was accompanied by overexpression of AD-related genes and their products: APP and BACE1 [23]. Similarly, enhanced taupathy and AD-like pathology were observed in aged primate brains decades after infantile exposure to lead [25]. In addition to those observations in animals exposed to Pb as infants, *in vitro* studies revealed that the exposure of differentiated neuroblastoma cells to lead induced a significant latent elevation in transcription factors Sp1 and Sp3, accompanied by a significant reduction in DNMT1, DNMT3A, and MeCP2 activities [26]. Others analyzed the DNA methylation pattern of the *APP* gene in rat pheochromocytoma (PC12) cells after Pb acetate exposure, observing induction of *APP* promoter demethylation, impaired DNMT1 expression, and changes in global DNA methylation levels [27].

Additional studies in cell cultures and animal models

Human neuroblastoma cells treated with anisomycin, an activator of stress-related proteins, showed increased intracellular Aβ levels and increased expression of APP, BACE1, and PSEN1 proteins resulting from demethylation of their gene promoters associated with the reduction of DNMT proteins [47]. Induction of oxidative stress in human neuroblastoma cells treated with hydrogen peroxide (H_2O_2) led to upregulation of HATs and downregulation of HDACs, reduction of DNMT activities, upregulated expression of APP and BACE1 proteins resulting from demethylation in their gene promoters, and a significant increase of intracellular Aβ levels [48]. Similarly, early-life stress in rats (maternal separation) led to significant increases in corticosterone levels, BACE1 expression (resulting from promoter demethylation), and Aβ levels [31]. Moreover, exposure to the Aβ peptide induced global DNA hypomethylation in murine cerebral endothelial cells but led to hypermethylation of the gene encoding neprilysin (NEP), one of the enzymes responsible for Aβ degradation, thus suppressing NEP expression in mRNA and protein levels [19]. Similarly, the exposure of human neuronal cell cultures to soluble Aβ oligomers inhibited cysteine uptake, causing a reduction in the DNA methylation potential (SAM/SAH ratio) in association with an increase in homocysteine levels and a global decrease in DNA methylation [28]. Epigenetic modifications of several genes were also observed in cell lines bearing one of the *APP* mutations leading to excessive Aβ production and early onset of AD, the so-called Swedish double mutant of *APP* [20]. In this regard, a recent study demonstrated that APP family member proteins are involved in SAM formation by methionine adenosyltransferase II alpha (MAT2A), and their knockdown in cell cultures modified *BACE1* and *PSEN1* gene expression, likely as a consequence of MAT2A deregulation [49].

DNA methylation changes in Alzheimer's disease: postmortem brains

Studies in postmortem brain specimens of AD subjects, of individuals with cognitive decline, and of healthy matched controls can be broadly divided into: (1) genome-wide approaches, aimed at evaluating the global content of 5-mC or 5-hmC, and (2) candidate-gene approaches, which look at the DNA methylation profiles of CpG islands within a single or a few genes, chosen on the basis of their plausible involvement in AD pathogenesis [6]. The author has recently reviewed those studies [6] that, albeit promising and indicative of possible epigenetic changes relevant to AD, are limited by the sample sizes of case-control cohorts (often a dozen of brains or less), by differences in storage and processing of postmortem materials, by the application of different techniques for DNA methylation analysis, by the presence of different cell types within the tissue analyzed, and by the fact that the analysis of postmortem AD brains means looking at the end stage of a degenerative process and does not allow for discrimination between early epigenetic changes from those arising as a consequence of the degenerative process itself [6]. As a result, those studies are often conflicting, and no single gene has unequivocally emerged as a replicated epigenetic marker of the disease.

Whole-genome approaches in postmortem brains

The analysis of global DNA methylation in postmortem brain regions of a rare set of monozygotic twins discordant for AD revealed reduced levels of DNA methylation in temporal neocortex nuclei of the AD twin, evaluated by means of immunocytochemistry for 5-mC content [32]. The same group analyzed postmortem brain tissues from 20 AD patients and 20 age-matched controls, and used immunocytochemistry for two markers of DNA methylation (5-mC and 5-methylcytidine) and eight methylation maintenance factors (DNMT1 and different components of the MeCP1/MBD2 methylation complex) in the entorhinal cortex layer II, again observing significant decrements in AD cases [33]. More recently, 5-methylcytidine and 5-hydroxymethylcytidine contents have been immunohistochemically investigated in the hippocampus of 10 AD patients and 10 matched controls. The results showed a robust decrease (almost 20%) in the hippocampal levels and a negative correlation with amyloid plaque load for both markers of DNA methylation in AD patients [34]. Others compared human postmortem frontal cortex genome-wide DNA methylation profiles between 12 AD and 12 cognitively normal matched subjects. The analysis revealed 948 CpG sites representing 918 unique genes. On average, only a subtle mean methylation difference (2.9%) between AD cases and controls was observed. The promoter of the gene coding for transmembrane protein 59 (*TMEM59*) showed a certain degree of difference between patients and controls, with a 7.3% reduced methylation in AD cases. The *TMEM59* gene is implicated in APP posttranslational processing [35]. By contrast, a study performed on 10 AD brains, 10 brains of patients with bipolar disorders (BDs), and 10 matched control brains revealed that AD and BD brains showed several epigenetic similarities, including global DNA hypermethylation in the frontal cortex, with respect to controls [36].

Candidate-gene approaches in postmortem brains

Several genes have been investigated for promoter methylation in postmortem brains of AD individuals [6]. Due to space allowance this section will discuss in detail only those papers showing significant differences between AD and healthy matched specimens. The DNA methylation status at 50 loci, encompassing primarily 5′ CpG islands of genes related to CNS growth and development, was analyzed in the temporal neocortex of 125 subjects ranging in age from 17 weeks of gestation to 104 years of age and including a cohort of AD patients [37]. Only two loci showed

AD-related changes: *SORBS3* and *S100A2* encoding a cell adhesion molecule and a calcium-binding protein, respectively [37]. Others observed hypermethylation of the nucleolar rRNA gene (*rDNA*), transcription of which is critical for ribosomal biogenesis, in cerebrocortical regions of mild cognitive impairment (MCI) and AD individuals [38]. MCI can be considered to be a transitional stage between normal aging and dementia, and, in the parietal cortex, the rDNA promoter was hypermethylated more in MCI than in advanced AD [38]. Both AD and BD frontal cortices (Brodmann area 9) have been investigated for promoter methylation of neuroinflammatory and neurotrophic genes. Particularly, 10 AD and 10 BD brains showed decreased methylation of the promoter of the cyclooxygenase 2 (*COX2*) gene compared with respective controls. *COX2* codes for a protein involved in neuroinflammatory processes, and the protein levels were increased in both AD and BD frontal cortex. AD brains also showed significantly decreased methylation of the neuroinflammatory factor NF-κB promoter region, which was accompanied by reciprocal increases in NF-κB p50 and p65 subunit mRNA expression [36]. By contrast, there was a significant decrease in brain-derived neurotrophic factor (BDNF) mRNA in the AD brain, and both the *BDNF* gene and the BDNF transcription factor *CREB* gene promoters were significantly hypermethylated in AD brains [36]. There was also a significant increase in DNA methylation at the promoter region of the gene coding for the synaptic protein synaptophysin in the AD brains, overall suggesting an epigenetic enhancement of neuroinflammatory processes and a repression of neuronal plasticity in AD frontal cortex [36]. Interestingly, despite some reported age-related epigenetic changes, no significant differences have been observed when comparing AD with control brains for key AD genes involved in Aβ formation and deposition (*APP*, *PSEN1*, *APOE*) or NFT formation (*MAPT*) [39,40].

DNA methylation changes in Alzheimer's disease: peripheral blood cells

In order to avoid the problems arising with recruitment, handling, storage, and processing of postmortem brain samples, researchers are currently analyzing blood DNA samples obtained from peripheral blood mononuclear cells (PBMCs) searching for more easily accessible DNA methylation biomarkers of AD. Such studies can be divided into those searching for global DNA methylation changes (by means of the analysis of DNA repetitive elements, such as Alu, LINE, and telomere sequences), and those following a candidate-gene approach.

DNA methylation analysis of repetitive elements in blood DNA

The methylation pattern of repetitive elements (Alu, LINE-1, and α-satellite DNA) was assessed in DNA obtained from PBMCs of 43 AD patients and 38 matched controls, and the study revealed higher levels of *LINE-1* methylation in AD subjects with respect to controls [41]. The methylation status of telomere regions was investigated in PBMCs of 34 AD patients and 49 healthy controls. The subtelomeres in the shortest telomeres were relatively more methylated in AD patients of both genders, compared with age-matched controls [42].

Markers of DNA methylation in blood DNA: candidate genes

An increase in fatty acid amide hydrolase (*FAAH*) and 5-lipoxygenase (*5-LOX*) gene expression and a reduction of their promoter methylation were observed in PBMCs of AD subjects with respect to age-matched controls [43,44]. The peptidyl-prolyl *cis*/*trans*-isomerase Pin1 acts on both tau and APP to regulate their functions by influencing tau phosphorylation and APP processing; the analysis of

PBMCs of AD subjects and matched controls revealed a statistically significant reduction in promoter methylation, paralleled by a significantly increased Pin1 expression in AD [45]. The analysis of promoter polymorphisms of the *DNMT3B* gene revealed no association with AD risk when the gene was evaluated independently from other factors [50]. However, when a panel of polymorphic genes involved in one-carbon unit transport and metabolism, SAM production, and DNA methylation reactions was evaluated simultaneously with circulating biochemical markers of B-vitamin status (serum folate, vitamin B_{12}, and plasma homocysteine), a combination of those biomarkers allowed discrimination with significant accuracy between AD patients and matched controls [51].

Histone modifications in Alzheimer's disease

Increasing evidence suggests that histone modifications also play a pivotal role in AD pathogenesis, and there is a lot of evidence demonstrating their close relationship with memory impairment [4–6]. Although studies on humans are scarce, studies performed on animal models and cell cultures revealed the contribution of histone modifications to learning and memory processes, and there is increasing evidence that the manipulation of histone acetylation with HDAC inhibitors (HDACi) in AD animal models often results in both prevention of cognitive deficits and memory recovery [6]. A summary of those studies is shown in Table 17.2.

Cell cultures and animal models

Studies in AD animal models suggest that impaired histone acetylation/deacetylation processes might be involved in memory impairment and AD. For example, Francis and coworkers [52] showed that after fear conditioning training the levels of hippocampal acetylated H4 in the *APP/PS1* mouse model of AD were about 50% lower than in wild-type littermates. However, inhibiting histone deacetylation prior to training rescued acetylated H4 levels to wild-type values and improved learning performances. It was also demonstrated that cognitive capacities in the neurodegenerating brain are constrained by an epigenetic blockade of gene transcription, mediated by HDAC2, that is potentially reversible. HDAC2 was increased by AD-related neurotoxic insults *in vitro*, in two mouse models of

Table 17.2 Evidence of Histone Modifications in Alzheimer's Disease and AD Model Systems

Experimental Model	Observation	References
AD animal models	Reduced levels of H3 and H4 acetylation	Francis et al. [52], Lithner [53]
	HDAC inhibitors improved cognition and memory functions	Gräff et al. [54], Govindarajan et al. [55], Karagiannis et al. [56]
Postmortem AD human brains	Decreased levels of H3 acetylation	Zhang et al. [57]
	Increased levels of H3 phosphorylation	Rao et al. [36], Ogawa et al. [58]
	Increased levels of HDAC6 and HDAC2	Gräff et al. [54], Ding et al. [59]

neurodegeneration, and in patients with AD, leading to chromatin condensation and reduced expression of genes important for learning and memory [54]. Reversing the build-up of HDAC2 unlocked the repression of those genes, reinstated structural and synaptic plasticity, and abolished neurodegeneration-associated memory impairments [54]. Similarly, reducing levels of endogenous HDAC6, a protein involved in tau phosphorylation and accumulation, restored learning and memory in a mouse model of AD [55]. Several other examples are available in the literature revealing the importance of histone acetylation in memory and suggesting that inhibiting histone deacetylation by means of HDAC inhibitors, such as trichostatin A, valproic acid, and phenylbutyrate, could represent a promising treatment for AD [56]. A comprehensive description of those studies is provided in Chapter 27 of this book.

Moreover, histone H3 homeostasis is disrupted in association with the appearance of soluble Aβ at an early stage in AD progression [53]. Histone H3 acetylation, phosphorylation, and methylation were analyzed in multiple model systems (neuroblastoma cell cultures, primary cortico/hippocampal neurons, AD mice, and AD neocortex), and both *in vitro* and *in vivo* studies indicated that multimeric soluble Aβ may be a potent signaling molecule indirectly modifying the transcriptional activity of DNA by modulating histone H3 modifications [53]. In this regard, it was shown that BACE1 mRNA levels were increased in the brain of the triple transgenic animal model of AD (3xTg AD mice) as well as in AD PBMCs along with an increase in promoter accessibility and histone H3 acetylation, while the BACE1 promoter region was less accessible in PBMCs from MCI individuals [60].

Postmortem brains

Changes in histone acetylation, phosphorylation, and methylation have also been observed in postmortem brains of AD individuals. For example, decreased levels of H3K18/K23 acetylation were observed in AD temporal lobe compared with matched controls [57], and increased levels of histone deacetylases, such as HDAC6 and HDAC2, have been reported in AD brain regions, the first leading to increased tau phosphorylation and the latter repressing several genes important for learning and memory capabilities [54,59]. Moreover, increased levels of H3 phosphorylation linked to chromatin compaction have been observed in AD brains [36,58].

DNA methylation changes and histone modifications in Huntington's disease

Huntington's disease (HD) is a progressive neurodegenerative disorder resulting in cognitive impairment, choreiform movements, and death, which usually occurs 15 to 20 years after the onset of symptoms. Affected individuals also show psychiatric and behavioral disturbances [61]. HD is caused by a CAG repeat expansion within exon 1 of the gene encoding huntingtin (*HTT*). In normal individuals, the number of CAG repeats is maintained below 35, but in HD subjects it ranges from 36 to more than 100, resulting in an expanded polyglutamine segment in the protein. The disease is inherited as an autosomal dominant trait, and age at onset of the symptoms is inversely correlated with the number of CAG repeats. A loss of function of normal huntingtin and/or a toxic gain of function of the mutant one contributes to the disruption of multiple intracellular pathways, ultimately leading to neurodegeneration [61]. In this regard, there is increasing evidence of interactions between huntingtin

Table 17.3 Some Examples of Epigenetic Modifications in Huntington's Disease and HD Model Systems

Experimental Model	Observation	References
Cell cultures expressing mutant huntingtin	Changes in gene expression and promoter methylation of several genes	Ng et al. [62]
Cell cultures expressing mutant huntingtin	Depletion of the HAT protein CBP or recruitment of CBP into huntingtin aggregates, and histone hypo-acetylation	Jiang et al. [63]
	Increased monoubiquitylation of H2A, inducing aberrant gene expression via altered histone methylation	Kim et al. [64] Bett et al. [65]
	Increased transcription of the ATRX chromatin remodeling protein, with subsequent trimethylation of H3 and condensation of pericentromeric chromatin	Lee et al. [66]
HD mouse models	Reduced CBP hippocampal expression and histone H3 hypo-acetylation leading to reduced expression of CBP target genes	Jiang et al. [63]
	Genome-wide reduction of H3 acetylation, linked to transcriptional dysregulation of several genes	Valor et al. [67] McFarland et al. [68]
HD models: mice, worms, flies	Treatment with HDACi reduced neurodegeneration and improved motor impairments	Gray [69] Giralt et al. [70]
Postmortem HD human brains	Changes in H3 trimethylation and histone acetylation	Vashishtha et al. [71] Yeh et al. [72]

and proteins regulating the histone code, suggesting that epigenetic mechanisms might also contribute to disease pathogenesis (Table 17.3) and that targeting the histone code might represent a potential therapeutic approach [69].

DNA methylation changes in Huntington's disease

Trinucleotide repeat expansion underlies several neurological diseases, including HD. In affected individuals, the expanded loci are characterized by dramatic changes in chromatin structure and repeat tract length, and some studies have identified alterations in DNA methylation, histone modification, and chromatin structure around the expanded repeat tract that are not present at the corresponding wild-type allele and might contribute to repeat instability [73,74]. However, a clear evidence of impaired DNA methylation in HD came from a study published in 2013 [62]. Researchers used cells expressing either mutant or wild-type huntingtin, observing that a large fraction of the genes that change in expression in the presence of mutant huntingtin demonstrate significant changes in DNA methylation [62]. More recently, others developed a method to detect N-7 guanine methylation, a mark that might be involved in transcriptional regulation even if its function is still not completely clarified, and observed significant changes in N-7 guanine methylation in nucleic acids obtained from human and transgenic mouse HD brains, with respect to control brains [75].

Histone modifications in Huntington's disease

There is substantial evidence that mutant huntingtin interacts directly with proteins regulating the histone code, including HATs, HDACs, and others [69]. Such evidence led researchers to use several disease models, including cell cultures, worms, fly models, and transgenic mice to address the contribution of histone modifications to disease pathogenesis [69]. Postmortem human brains were screened to confirm changes of the histone code in affected brain regions, and recent genome-wide studies in HD mice have been performed to investigate whether changes in histone modifications correlate with transcriptional abnormalities across the entire transcriptome [67].

Cell cultures and animal models

Mutant huntingtin interacts directly with the histone acetyltransferases KAT3A (CREB-binding protein, CBP) and KAT2B (P/CAF), and this complex interacts with p53 and represses transcription of the p53-regulated promoters in cell cultures [76]. Transfection of mutant huntingtin in HT22 cells or primary neurons caused cell toxicity and either depletion of CBP or its recruitment into huntingtin aggregates, and this was accompanied by histone hypo-acetylation [63]. In this regard, it was recently demonstrated that impairment of long-term spatial and recognition memory in heterozygous HD knock-in mutant mice "Hdh(Q7/Q111)," a genetically accurate HD mouse model, is associated with reduced hippocampal expression of CBP and diminished levels of histone H3 acetylation [70]. In agreement with reduced CBP, the expression of CREB/CBP target genes related to memory was significantly reduced in the hippocampus of HD mice compared with wild-type mice. Finally, and consistent with a role of CBP in cognitive impairment, administration of the histone deacetylase inhibitor trichostatin A rescued recognition memory deficits and transcription of selective CREB/CBP target genes in HD mice [70]. Additional support of the importance of histone deacetylases in HD comes from several studies on HD animal models (*Caenorhabditis elegans*, *Drosophila*, and mice) where targeting of HDACs with HDACi reduced the associated neurodegeneration and improved motor impairments [69].

A recent genome-wide histone acetylation study in striata of the transgenic R6/2 HD mouse model revealed genome-wide decreases in histone H3 acetylation at lysine residues 9 and 14 (H3K9 and H3K14), compared with control mice, and that histone H3 acetylation and active gene expression are intimately tied in the rodent brain [68]. A similar recent study examined the genome-wide correlation of histone acetylation and gene expression in a mouse model of early-onset HD, revealing hundreds of loci that were hypo-acetylated for H3K9, H3K14, and H4K12 in the animal brain. Particularly, this screen identified a subset of genes in which H3K9 and K14 deacetylation and transcriptional dysregulation concur. Genes in this group were consistently affected in different brain areas, mouse models, and tissue from patients, suggesting that histone deacetylation and transcriptional dysregulation might be two early manifestations of the disease [67].

Mutant huntingtin also affects histone monoubiquitylation [64]. Disrupted interaction of huntingtin with Bmi-1, a component of the ubiquitin ligase complex, increases monoubiquityl histone H2A (uH2A) levels in a cell culture model of HD [64], and the levels of uH2A were significantly increased in the brains of R6/2 mice [65]. The functional consequence of this increase is an aberrant gene expression via altered histone methylation [64]. Genes with expression that is repressed in transgenic R6/2 mouse brain have increased uH2A and decreased uH2B at their promoters, whereas actively transcribed genes show the opposite pattern. Reduction in uH2A reverses transcriptional repression

and inhibits methylation of histone H3K9 in cell culture. In contrast, reduction in uH2B induces transcriptional repression and inhibits methylation of histone H3K4 [64].

It was also observed that mutant huntingtin induces the transcription of the chromatin remodeling protein ATRX (alpha thalassemia X-linked mental retardation), whose expression was elevated in both a cell-line model and a transgenic model of HD and induced trimethylation of H3K9 and condensation of pericentromeric heterochromatin. Knock-down of ATRX decreased H3K9 methylation and improved the hatch rate of transgenic HD fly embryos, but its overexpression exacerbated eye degeneration in the flies [66].

Interestingly, CAG/CTG expansions are unstable in somatic tissues, and there is a tendency toward a further somatic expansion of the inherited expanded alleles that accelerates disease progression. Although the mechanisms underlying repeat instability are complex, a study performed in HD R6/2 mice suggests that tissue-specific instability of CAG/CTG repeats might be partially regulated by chromatin accessibility at the HD locus and by specific histone modifications, such as H3K26 trimethylation, a mark associated with transcription elongation [77]. Furthermore, CAG/CTG repeats alter the affinity for the histone core and the positioning of DNA in the nucleosome [78].

Postmortem brains

Studies in postmortem brains of affected individuals confirmed most of the changes in histone modifications observed in disease models, such as histone deacetylation and increased trimethylation of H3 [67,69,79]. For example, gene-specific changes in histone H3K4 trimethylation were observed at transcriptionally repressed promoters in both R6/2 mice and human HD brains [71], and a recent immunohistochemical analysis showed specific and significant losses of H2A, H2B, H3, and H4 acetylation from cells in the caudate nucleus and from Purkinje cells of the cerebellum in HD compared with patients with frontotemporal lobar degeneration and control subjects, while the level of HDAC5 was increased in these cells [72]. A dynamic regulator of chromatin plasticity, H2A histone family member Y (H2AFY) is specifically overexpressed in the blood and frontal cortex of patients with HD compared with controls. This association precedes the onset of clinical symptoms, was confirmed in two mouse models, and was independently replicated in cross-sectional and longitudinal clinical studies [80].

DNA methylation changes and histone modifications in Parkinson's disease

Parkinson's disease (PD), the second most common neurodegenerative disorder after AD, is clinically characterized by resting tremor, rigidity, bradykinesia, and postural instability as well as non-motor symptoms such as autonomic insufficiency, cognitive impairment, and sleep disorders. Pathologically, PD is characterized by progressive and profound loss of neuromelanin containing dopaminergic neurons in the substantia nigra with the presence of eosinophilic, intracytoplasmic inclusions called Lewy bodies (LBs), which contain aggregates of α-synuclein as well as other substances, and Lewy neurites in surviving neurons. Although some improvements of the symptoms can be achieved with levodopa and dopaminergic therapy, there is no available treatment to arrest the progression of the disease. The majority of PD cases are sporadic, likely arising from a combination of polygenic inheritance, environmental exposures, and complex gene–environment interactions superimposed on slow

and sustained neuronal dysfunction due to aging [81]. A familial history of PD is shown in approximately 20% of cases, and in a minority of them the disease is inherited as a Mendelian trait. Studies in PD families allowed the identification of at least 15 PD loci, named sequentially PARK1–15, and several causative genes have so far been identified or hypothesized. Although follow-up genetic studies are inconsistent for some of them or conclusive data are still pending, there is evidence that five of those genes—*α-synuclein* (*SNCA*), *parkin* (*PARK2*), *PTEN-induced putative kinase 1* (*PINK1*), *DJ-1*, and *leucine-rich repeat kinase 2* (*LRRK2*)—cause typical PD [82].

Most of the studies concerning a possible contribution of DNA methylation to PD pathogenesis have focused on the analysis of promoter methylation of key PD genes in postmortem brains and PBMCs of affected individuals [83]. By contrast, our knowledge of histone modifications in PD derives mainly from studies on cell cultures and animal models of the disease, such as those induced by mitochondrial toxins, including 1-methyl-4-phenylpyridinium (MPP +), paraquat, and rotenone, or those overexpressing human α-synuclein [83].

DNA methylation changes in Parkinson's disease: cell cultures and animal models

Some studies investigating the possible contribution of DNA methylation in neuronal cell cultures and animal models of PD have been performed following the observation that α-synuclein sequesters DNMT1 from the nucleus [84]. DNMT1 is the maintenance DNA methylation enzyme that is abundantly expressed in the adult brain and is mainly located in the nuclear compartment. A reduction of nuclear DNMT1 levels was observed in human postmortem brain samples from both PD individuals and patients with dementia with Lewy bodies (DLB), as well as in the brains of *α-synuclein* transgenic mice. Furthermore, sequestration of DNMT1 in the cytoplasm resulted in global DNA hypomethylation in human and mouse brains, involving CpG islands upstream of *SNCA* and other genes [84]. The nuclear DNMT1 levels were partially rescued by overexpression of DNMT1 in neuronal cell cultures and in α-synuclein transgenic mice brains, suggesting that α-synuclein might induce aberrant subcellular localization of DNMT1, resulting in epigenetic modifications in the brain [84].

The DNMT inhibitor 5-aza-2′-deoxycytidine (5-aza-dC) was tested in cultured dopaminergic neurons. Neurotoxicity of MPP+, 6-hydroxydopamine, or rotenone was also assayed with 5-aza-dC pretreatment. 5-aza-dC resulted in decreased cell viability and increased apoptosis in dopaminergic neuronal cells. Pretreatment with 5-aza-dC exacerbated neurotoxic damage to dopaminergic neurons induced by MPP+, 6-hydroxydopamine, or rotenone. 5-aza-dC and MPP+ also induced transcriptional promoter demethylation and upregulation of the gene coding for α-synuclein [85]. A similar study was performed with PC12 cells pretreated with 5′-aza-dC and then exposed to paraquat [86]. The study revealed that the demethylating agent 5′-aza-dC sensitizes paraquat toxic effects on PC12 cells by exacerbating mitochondrial deficit and oxidative stress [86].

DNA methylation changes in Parkinson's disease: individuals

Postmortem brains: from candidate genes to genome-wide studies

Several investigators assessed the methylation status of the *SNCA* gene in postmortem brain regions of PD individuals. A reduced methylation pattern of *SNCA* promoter and intron1 was observed in several brain regions of sporadic PD patients including the *substantia nigra*, likely resulting in increased mRNA and protein levels [87,88]. Moreover, as discussed in the previous section, α-synuclein

sequesters DNMT1 from the nucleus into the cytoplasm, and reduced DNMT1 levels were observed in postmortem PD brains [84].

Mutations of *parkin* cause autosomal recessive juvenile parkinsonism, which differs from conventional PD in that there is usually no LBs formation. Studies in families with autosomal recessive forms of PD have highlighted a central role for mitochondrial damage, repair, and turnover in PD pathogenesis, with parkin, DJ-1, and PINK1 participating in overlapping mitochondrial pathways [82]. The analysis of postmortem brains revealed no difference in *PARK2* methylation between PD patients and controls [89].

Recent genome-wide approaches combining methylation with gene expression levels revealed DNA hypomethylation and increased mRNA expression of cytochrome P450 2E1 (*CYP2E1*) in PD brains with respect to controls [90]. Moreover, a large dataset of postmortem brain samples revealed changes in methylation and gene expression associated with PD risk variants in *PARK16*, transmembrane glycoprotein NMB (*GPNMB*), and syntaxin 1B (*STX1B*) genes [91]. Interestingly, a recent study of genome-wide DNA methylation in brain and blood samples from PD patients revealed a distinctive pattern of methylation involving many genes previously associated with PD. The study identified concordant methylation alterations in brain and blood, suggesting that blood might represent a good surrogate for brain tissue to detect DNA methylation in PD [92].

Peripheral blood cells

The study of the methylation status of seven major human clock genes involved in circadian rhythm—*PER1*, *PER2*, *CRY1*, *CRY2*, *Clock*, *NPAS2*, and *BMAL1*—revealed that the methylation frequency of the *NPAS2* promoter was significantly decreased in PD patients with respect to healthy matched controls [93]. The analysis of subtelomeric methylation patterns in PBMCs revealed that PD patients had fewer short telomeres than healthy controls. Moreover, short telomeres in PD patients showed a constant methylation pattern, and an age-related demethylation of short telomeres was observed in controls [94].

Histone modifications in Parkinson's disease

Little is known concerning histone modifications in PD brains, and most of our knowledge concerning histone modifications in PD derives from *in vitro* studies in cell cultures or research employing animal models of the disease, such as those induced by the mitochondrial MPP+, paraquat, and rotenone, or those overexpressing human α-synuclein [95–98]. The author recently reviewed the literature in the field, and a summary of those studies is provided in Tables 17.4 and 17.5).

Studies performed in mice and *Drosophila* models of the disease revealed that α-synuclein translocates into the nucleus, binds with histones, and inhibits histone acetylation [95,96]. The toxicity of α-synuclein was rescued by the administration of HDACi, which counteracted α-synuclein translocation into the nuclei, resulting in increased H3 acetylation in several models of PD, including mice and neuronal cell cultures obtained with the administration of mitochondrial toxins, such as rotenone, paraquat, or MPP+ [97,98].

There are also indications that histone deacetylase 6 (HDAC6) is concentrated in LBs in PD and dementia, and was shown to play a critical role in the protection of dopaminergic neurons and promoted the formation of α-synuclein inclusions in mice and *Drosophila*. It was therefore suggested that the accumulation of HDAC6 might be specific to α-synucleinopathy and that LBs might

Table 17.4 Some Examples of DNA Methylation Changes in Parkinson's Disease and PD Model Systems

Experimental Model	Observation	References
Neuronal cell cultures and rodents	α-Synuclein sequesters DNMT1 in the cytoplasm, resulting in reduced nuclear DNMT1 levels and global hypomethylation.	Desplats et al. [84]
Neuronal cell cultures	Pretreatment with the DNA demethylating agent 5-aza-2′-deoxycytidine sensitizes neuronal cell cultures to the toxic effects of MPP+, rotenone, and paraquat and induces *SNCA* promoter demethylation and increased gene expression.	Wang et al. [85] Kong et al. [86]
Postmortem PD human brains	Reduced methylation of the *SNCA* promoter and increased α-synuclein production	Jowaed et al. [87] Matsumoto et al. [88]
	Reduced DNMT1 levels	Desplats et al. [84]
	Reduced methylation of the *CYP2E1* promoter and increased mRNA levels	Kaut et al. [90]
	Changes in the methylation profiles of other genes, including *PARK16*, *GPNMB*, *STX1B*	IPDGC and WTCCC2 [91]
Human PD blood cells	Increased methylation of subtelomeric regions of the short telomeres	Maeda et al. [94]
	Reduced methylation of the clock genes *NPAS2*	Lin et al. [93]

Table 17.5 Evidence of Histone Modifications in Parkinson's Disease and PD Model Systems

Experimental Model	Observation	References
Disease models: cell cultures, flies and mice	α-Synuclein translocates into the nucleus, binds to histones, and inhibits histone acetylation.	Goers et al. [95] Kontopoulos et al. [96]
	HDACi administration counteracts α-synuclein translocation into the nuclei, resulting in increased histone H3 acetylation.	Outeiro et al. [97] Harrison and Dexter [98]
Disease models: flies and mice	HDAC6 promotes the formation of α-synuclein inclusions and might represent a cytoprotective response to sequester toxic proteins.	Richter-Landsberg and Leyk [99]
Human neuroblastoma cells	PINK1 interacts with proteins involved in histone methylation (EED/WAIT1) leading to reduced trimethylation of H3K27.	Berthier et al. [100]

represent cytoprotective responses to sequester toxic proteins, such as α-synuclein [99]. A recent study in human neuroblastoma cells revealed that PINK1, whose mutations are associated with early-onset recessive forms of PD, interacts with the embryonic ectoderm development polycomb histone-methylation modulator (EED/WAIT1), and the interaction resulted in a reduced trimethylation of H3K27, which affected polycomb-regulated gene transcription [100].

Conclusions

Epigenetic marks, such as those described in this chapter, play a fundamental role in the neuronal plasticity required for learning and memory. As a consequence, changes of the epigenetic landscape can contribute to the age-related cognitive decline as well as to cognitive impairment and neurodegeneration. Studies on rodents, primates, and neuronal cell cultures have shown that the environment plays a fundamental role in determining long-lasting epigenetic changes. For example, early-life exposure to lead was shown to induce epigenetic changes in the aged brain of rodents and monkeys, and early-life stress in rats (maternal separation) induced epigenetic changes of AD-related genes. Dietary factors, such as B-vitamin deprivation, are among other modulators of the epigenome, as they are able to induce changes in DNA methylation and gene expression in mice, leading to Aβ overproduction and accumulation, resulting in brain changes resembling those of AD. Similarly, the neurotoxic effects of MPP+, rotenone, and paraquat are counteracted by epigenetic drugs in animal models of PD. It is noteworthy that there is increasing evidence suggesting that many of the causative genes of Mendelian forms of neurodegenerative diseases code for products interacting with the epigenetic landscape. Some examples are the Aβ peptide that induces global DNA hypomethylation in neuronal cell cultures, the α-synuclein protein that binds to DNMT1 and sequesters it into the cytoplasm, PINK1 that interacts with proteins required for histone methylation, or the huntingtin protein that directly interacts with several proteins regulating the histone code. Overall, these examples support the notion that global changes of the epigenome occur either in familial or sporadic forms of neurodegeneration and might be responsible for several of the symptoms accompanying those disorders. This notion is largely supported by studies in disease animal models showing that epigenetic drugs such as HDACi ameliorated several cognitive and motor symptoms, thereby opening a window for potential therapeutic interventions.

There is increasing interest in understanding how changes in DNA methylation and histone modifications are reflected by those in gene expression and protein levels. Unfortunately, given the scarce availability of postmortem brain tissues and the difficulty in evaluating life-long environmental exposures in those samples, such studies have been mainly performed on rodents and cell cultures, and investigators are now searching for easily detectable surrogate epigenetic biomarkers in PBMCs of affected individuals to be correlated with both exposure biomarkers and changes in gene expression. The future will involve the integration of the exposome and the proteome with data on genome-wide epigenetic and gene expression profiles, as well as a deeper understanding of the possible contribution of drugs, dietary factors, and lifestyles to mold the epigenome, preventing or counteracting cognitive decline and neurodegeneration.

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Glossary

Alzheimer's disease The most common neurodegenerative disorder and the primary form of dementia in the elderly. Neurodegeneration in Alzheimer's disease leads to memory loss accompanied by changes of behavior and personality severe enough to affect daily life.

DNA methylation The addition of a methyl group (CH3) to the 5′ position of the cytosine pyrimidine ring, forming 5-methylcytosine (5-mC), a mark that in the promoter region is usually associated with gene silencing.

Histone modifications Covalent posttranslational modifications of amino-acidic residues on the histone tails, such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. The combination of those modifications, and their interplay with DNA methylation and chromatin remodeling proteins, regulates the chromatin structure in a dynamic fashion.

Huntington's disease Progressive neurodegenerative disorder resulting in cognitive impairment, choreiform movements, and psychiatric and behavioral disturbances.

Parkinson's disease The second most common neurodegenerative disorder after Alzheimer's disease, clinically characterized by resting tremor, rigidity, bradykinesia, and postural instability as well as non-motor symptoms such as autonomic insufficiency, cognitive impairment, and sleep disorders.

CHAPTER

Epigenetics in Intellectual Disability

18

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KEY CONCEPTS

- Epigenetic processes are disrupted in many forms of ID.
- ID can result from aberrant epigenetic silencing of important neuronal genes or from mutations in genes that directly modify chromatin structure.
- Fragile X syndrome is caused by aberrant silencing of the *FMR1* gene.
- Angelman and Prader–Willi syndromes are caused by the combined effects of 15q11q13 deletions and genomic imprinting.
- DNA methylation is an important epigenetic mechanism in learning and memory, and abnormalities involving DNA methylation and the methyl-DNA-binding protein, MeCP2, can cause ID.
- Disruption of the ATP-dependent chromatin remodeling complexes causes ID.
- Disruption of histone modification complexes causes ID.
- Many epigenetic ID genes encode proteins that act in common pathways or complexes that may be important for cognition.

ABBREVIATIONS

3C Chromosome conformation capture
5hmC 5-Hydroxymethylcytosine
5mC 5-Methylcytosine
Ac Acetylation
ACTB Actin, beta
ACTL6A/B Actin-like 6 A/B
ADD ATRX–DNMT3–DNMT3L
ARID AT rich interactive domain
AS Angelman syndrome
ASH2L ASH2-like
AST Antisense transcript
ASXL1 Additional sex combs like 1
ATP Adenosine triphosphate
ATP10C ATPase, Class V, type 10C
ATRX Alpha thalassemia X-linked mental retardation
BAF Brg1-associated factor
BDNF Brain-derived neurotrophic factor
BWS Beckwith–Wiedeman syndrome
CHARGE Coloboma, heart anomaly, atresia of the nasal choanal, retardation, and genital and ear anomalies
CHD Chromodomain helicase DNA-binding protein
ChIP Chromatin immunoprecipitation assay
COMPASS Complex of proteins associated with Set1
CREB1 cAMP responsive element-binding protein 1
CREBBP CREB-binding protein
CREST $Ca2^{+}$-responsive transactivator
CTCF CCCTC-binding factor
CUL4B Cullin 4B
CXXC1 CXXC finger protein 1
DEAD/H Asp–Glu–Ala–Asp box helicase
DNMT DNA methyltransferase
DNMTi DNA methyltransferase inhibitor
DPY30 Dpy-30 homolog
EHMT Euchromatin histone methyltransferase
EZH2 Enhancer of zeste homolog 2
FMR1 Fragile X mental retardation gene 1
FXS Fragile X syndrome

FXTAS Fragile X-associated tremor/ataxia syndrome
H3K9me Histone H3 lysine 9 methylation
HCFC1 Host cell factor C1
HDAC Histone deacetylase
HDACi Histone deacetylase inhibitor
HP1 Heterochromatin protein 1
IC Imprinting center
ICF1 Immunodeficiency-centromeric instability 1
ID Intellectual disability
Igf2 Insulin-like growth factor 2 gene
INO80 Chromatin-remodeling ATPase INO80
ISWI Imitation SWI
JARID1C Jumonji/ARID domain-containing protein 1C
KANSL1 KAT8 regulatory NSL complex subunit 1
KAT Lysine acetyltransferase
KDM Lysine demethylase
KMT Lysine methyltransferase
MAGEL2 MAGE-like 2
MAPK Mitogen-activated protein kinase
me Methylation
MeCP2 Methyl-CpG-binding protein 2
MEN1 Multiple endocrine neoplasia
MIM Mendilian inheritance in man
MKRN3 Makorin ring finger protein 3 gene
MLL Mixed-linkage leukemia
MRD Mental retardation, autosomal dominant
MRXS Mental retardation, X-linked, syndromic
nBAF Neuronal BAF complex
NCOA6 Nuclear receptor coactivator 6
NCOR1 Nuclear receptor co-repressor 1
NDN Necdin gene
NIPBL Nipped-B-like protein
NPAP1 Nuclear pore associated protein 1
NSD1 Nuclear receptor-binding SET domain protein 1
NSL Non-specific lethal
NuRD Nucleosome remodeling deacetylation
PAGR1 PAXIP1-associated glutamate-rich protein 1
PAXIP1 PAX interacting protein 1
PHD Plant homeodomain
PHF PHD finger protein
PRC2 Polycomb repressive complex 2
PR-DUB Polycomb repressive deubiquitination
PWS Prader–Willi syndrome
RBBP5 Retinoblastoma-binding protein 5
RCOR1 REST co-repressor 1
RTT Rett syndrome
SBBYSS Say–Barber–Biesecker–Young–Simpson syndrome
SET Su(var)3-9, Enhancer-of-zeste, Trithorax
SIN3A SIN3 transcription regulator family member A
SIRT1 Sirtuin 1
SKI V-ski avian sarcoma viral oncogene homolog
SMARC SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
SMC Structural maintenance of chromosomes
SNRPN Small nuclear ribonucleoprotein polypeptide
snoRNAs small nucleolar RNAs
SNURF SNRPN upstream reading frame
SRCAP SNF2-related CBP activator protein
SWI/SNF SWItch/Sucrose Non-Fermentable
SWR1 SNF2 family helicase Swr1
UBE2A Ubiquitin protein ligase E2A
UBE3A-AST UBE3A antisense transcript
UTR Untranslated region
UTX Ubiquitously transcribed tetratricopeptide repeat, X chromosome
WDR WD repeat domain
YB1 Major histocompatibility complex, class II, Y box-binding protein I; DNA-binding protein B
YY1 Yin Yang 1 transcription factor

Introduction

Intellectual disability (ID) is the current term used to describe individuals with early-onset (pre-adult) limitations in intellectual functioning and adaptive behavior. By definition, individuals with ID have an IQ lower than 70 and have difficulties with day-to-day practical and social skills. The etiology of ID is diverse, including both environmental and genetic factors. Genetic aberrations contributing to ID range from large cytogenetically visible chromosomal abnormalities that affect many genes (e.g., trisomy 21) to single gene defects. Currently, more than 500 genes contribute to the etiology of monogenic ID (A. Schenck, pers. comm.), and analysis of these single gene defects has revealed

some common cellular pathways and processes that are disrupted [1]. Prominent examples of these include genes encoding synaptic proteins and proteins involved in Ras/MAPK signaling. This chapter focuses on another functional category that is emerging in ID: genes encoding regulators of chromatin structure, or epigenetics.

Definition of epigenetics

Epigenetics has recently been defined as "long-lived and reversible modifications to nucleotides or chromosomes that do not change the (DNA) sequence but can alter gene expression and phenotypes" [2]. Epigenetic modifications include DNA methylation, some histone modifications, the binding of non-coding RNA to DNA, alterations to nucleosome positioning, and irregular chromosome structure organization. Epigenetic regulators are the proteins or non-coding RNAs that contribute to these processes.

Types of epigenetic disruption in ID

Epigenetic processes can contribute to ID in two fundamentally different ways. First, through abnormal epigenetic regulation of specific genes or chromosomal regions and, second, through mutations that disrupt specific epigenetic regulators. Abnormal epigenetic silencing occurs in the nucleotide repeat disorder fragile X syndrome and in the imprinting disorders Angelman syndrome and Prader–Willi syndrome. In these disorders, underlying DNA changes result in an altered epigenetic state leading to the silencing of genes that are otherwise transcribed. Such unscheduled gene silencing can be deleterious for normal brain function. In the second scenario, single gene mutations affecting epigenetic regulators can result in disruption of normal epigenetic regulation in the brain. Currently, about 30 epigenetic regulators involved in DNA methylation, nucleosome remodeling, or histone modification are implicated in various monogenic forms of ID. In some cases, the disrupted genes encode proteins that have a known function in neuronal processes such as learning and memory; however, in many cases, the neuronal function for these genes is not known. This chapter will discuss our current understanding of epigenetic processes that are disrupted in ID disorders resulting from anomalous epigenetic modulation of ID gene expression or from monogenic disruption of epigenetic regulators.

Abnormal epigenetic silencing in ID

Fragile X syndrome is caused by trinucleotide repeat expansion and abnormal epigenetic silencing of *FMR1*

Fragile X syndrome (FXS) is one of the most common forms of ID, affecting about 1 in 5000 males. FXS is caused by silencing of the fragile X mental retardation 1 (*FMR1*) gene, which encodes a mRNA-binding protein that is essential for normal synaptic plasticity. *FMR1* is one of the first ID genes identified, over 20 years ago, and the genetic and functional characterization of this gene has been extensively reviewed [3]. The most common DNA mutation causing FXS involves the expansion of a trinucleotide (CGG) repeat in the 5′-UTR of *FMR1*. In the normal population, the number of repeats varies from 6 to 54. FXS occurs when the number of repeats exceeds 200, and this extension

of the repeat length is referred to as the full mutation. Individuals with repeat numbers between 55 and 200 (referred to as the premutation) do not have ID but can acquire other phenotypes with variable penetrance, including ovarian insufficiency and late-onset tremors and ataxia (known as fragile X-associated tremor/ataxia syndrome, or FXTAS). The presence of a large number of repeats in full mutation carriers is usually associated with hypermethylation of CpG sites within the repeats and into the first exon of *FMR1* (Figure 18.1). This epigenetic alteration causes transcriptional silencing of *FMR1* and the development of FXS [4]. In addition to increased DNA methylation, FXS individuals also have decreased acetylation of histones H3 and H4 and increased methylation of histone H3 on lysine 9 (H3K9me) in the *FMR1* regulatory region [5]. More recently, it was shown that the histone modifications H3K9me3 and H4K20me3 that mark constitutive heterochromatin are highly concentrated in the FXS repeat region. In contrast, the repressive histone marks H3K9me2 and H3K27me3, which are present in facultative heterochromatin, display a broader distribution over the *FMR1* gene [6]. This suggests that the extended repeat may act as a nucleating site for the heterochromatinization of *FMR1*. This altered epigenetic state likely limits access of the normal transcriptional machinery that is required for induction and maintenance of *FMR1* expression; however, the mechanism underlying repeat mediated epigenetic changes is not understood.

Restoration of *FMR1* expression via epigenetic intervention has been suggested as a means of therapy because the extended trinucleotide repeat does not affect the promoter or coding sequence of the *FMR1* gene. Indeed, treatment with the DNA methyltransferase inhibitor (DNMTi) 5-aza-2′-deoxycytidine (azadC) can lead to reactivation of *FMR1* in cell culture [7]. However, to use this as a potential treatment for FXS is problematic due to the toxicity of this compound and the fact that reversal of DNA methylation in this way requires cell division and would therefore not be effective in terminally differentiated neurons. Another approach has been to target histone deacetylases (HDACs) that deacetylate the *FMR1* regulatory region in the presence of the full mutation. The HDAC inhibitors (HDACi) trichostatin A and sodium butyrate, which target class I, II, and IV HDACs, did not show a strong reactivation of *FMR1* expression, but inhibition of the class III HDAC sirtuin 1 (SIRT1) did [5,8]. Restoration of *FMR1* expression through SIRT1 inhibition coincides with increased H3K16 acetylation at the FMR1 regulatory region and is dependent on the H3K16 acetyltransferase KAT8 (previously known as MOF) [8]. H3K16Ac was also shown to increase upon treatment with DNMTi, suggesting that H3K16 deacetylation occurs downstream of DNA methylation in the silencing process. In contrast, SIRT1 inhibition did not affect *FMR1* DNA methylation levels, thus demonstrating that restoration of *FMR1* gene expression does not require DNA demethylation *per se* (Figure 18.1). Taken together, it appears that DNA methylation of the *FMR1* full mutation is a precursor for H3K16 deacetylation, which is essential for *FMR1* silencing. Restoration of *FMR1* expression by targeting class III HDACi is a potential approach for the treatment of FXS; however, it still requires validation in the context of a whole organism model system.

Genomic imprinting of the chromosome 15q11q13 region in Prader–Willi and Angelman syndromes

Genomic imprinting refers to the parent-of-origin-specific epigenetic silencing of one allele of a gene. Imprinted genes are expressed from either the maternal or paternal chromosome and usually occur in clusters spanning several kilobases of DNA. Typically, these clusters contain both maternally expressed and paternally expressed genes. Many imprinted gene clusters contain an imprinting center

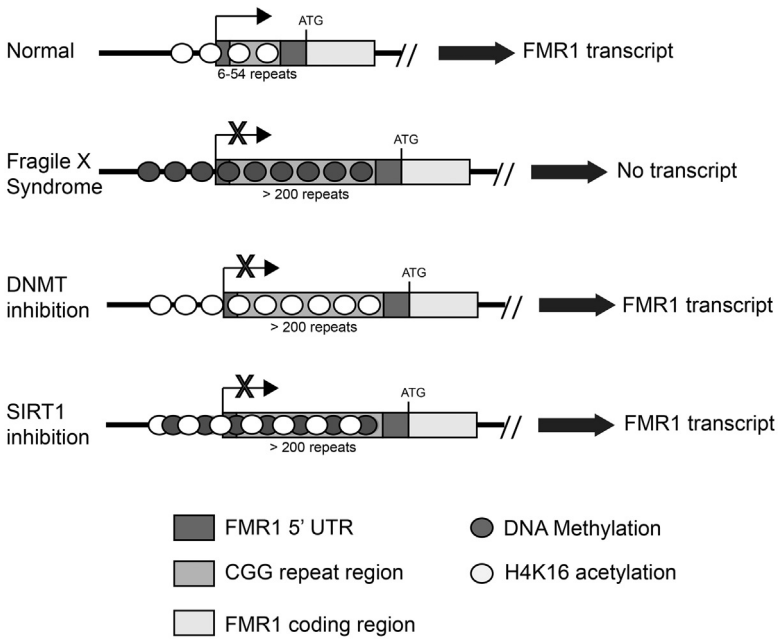

FIGURE 18.1

Abnormal epigenetic silencing of *FMR1* and restoration of *FMR1* expression with DNMT and HDAC inhibitors. Normal individuals have 6–54 trinucleotide repeats in the *FMR1* untranslated region (UTR). Individuals with fragile X syndrome (FXS) have greater than 200 repeats. FXS individuals have abnormal DNA methylation and lack of H4K16 acetylation in the *FMR1* regulatory region, leading to a lack of *FMR1* transcription. Inhibition of DNA methyltransferases (DNMTs) in FXS cells causes reduced DNA methylation of the *FMR1* regulatory region, restoration of H4K16 acetylation, and *FMR1* transcript expression. Inhibition of the class III HDAC SIRT1 restores H4K16 acetylation and *FMR1* expression but does not reduce DNA methylation in the *FMR1* regulatory region.

(IC) that is essential for imprinting of the cluster. ICs usually exhibit parent-of-origin-specific methylation and are often located at the promoter of a non-coding RNA that can mediate imprinting of the maternal or paternal allele in *cis*.

Two ID disorders linked to genomic imprinting are Prader–Willi syndrome (PWS) and Angelman syndrome (AS). The genetic basis of these disorders has been reviewed extensively [9]. These syndromes are clinically distinct and very recognizable; however, nearly identical deletions in the chromosome 15q11q13 region are associated with both disorders. In fact, this region contains a cluster of imprinted genes that are expressed from either the maternal or paternal chromosome and the clinical phenotype depends on the parental origin of the deletion (Figure 18.2). About 70% of PWS cases are caused by deletion of the paternal 15q11q13 region, which leads to loss of expression of the paternally expressed genes in this region. Similarly, about 70% of AS cases are caused by deletion of the maternal 15q11q13 region, leading to loss of expression of the maternally expressed genes. PWS and AS can also be caused by uniparental disomy for chromosome 15 in which PWS individuals (about

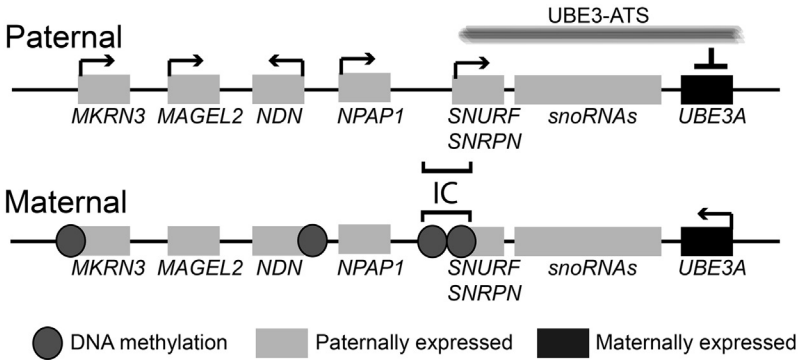

FIGURE 18.2

Schematic representation of the imprinted 15q11q13 region implicated in Angelman and Prader–Willi syndrome. Paternal expression of the UBE3A antisense transcript (UBE3A-AST) leads to *in cis* repression of the paternal *UBE3A* gene. *UBE3A-AST* expression is initiated from the *SNURF–SNRPN* promoter, which is the location of the 15q11q13 imprinting center (IC). This IC is maternally methylated, suppressing UBE3A-AST expression and allowing for maternal *UBE3A* expression. Other paternally expressed genes such as *MKRN* and *NDN* are repressed on the maternal allele through maternal methylation of the promoter.

25–30% of cases) have two copies of the maternal chromosome 15 and AS individuals (2–5% of cases) have two copies of the paternal chromosome 15.

The 15q11q13 region contains two maternally expressed genes, *UBE3A* and *ATP10C* (Figure 18.2). The paternal imprinting of *UBE3A* is tissue specific, with biallelic expression observed in most tissues and paternal silencing observed in neurons [10]. Interestingly, mutations in the *UBE3A* gene occur in about 10% of AS cases [9,11,12]. The AS phenotype is therefore caused by loss of *UBE3A* in neurons, whether this loss is mediated by point mutations, maternal 15q11q13 deletions, or uniparental disomy for the paternal chromosome 15. The situation for PWS is more complicated. The 15q11q13 region contains several paternally expressed genes, including *MKRN3*, *MAGEL2*, *NDN*, *NPAP1, SNURF–SNRPN*, and several small nucleolar RNA genes. There is no convincing evidence that implicates one of these paternally expressed genes in the full PWS phenotype.

A small proportion of AS and PWS cases show biparental inheritance of a normal chromosome 15. These cases are said to result from imprinting defects. In PWS, imprinting defects occur when maternal imprinting is observed on the paternal chromosome, leading to silencing of the paternally expressed genes. In AS, imprinting defects occur when paternal imprinting of the maternal chromosome leads to silencing of *UBE3A*. In rare cases these imprinting defects result from microdeletions in the critical 15q11q13 imprinting center located in the promoter and exon 1 of the *SNURF–SNRPN* gene (Figure 18.2) [9]. The 15q11q13 IC is normally methylated on the maternal allele leading to silencing of expression from the *SNURF–SNRPN* promoter. This promoter is very interesting because not only does it control expression of *SNURF–SNRPN* but it also produces a long read-through transcript encompassing several small nucleolar RNAs (snoRNAs) followed by a *UBE3A* antisense transcript (*UBE3A–AST*). The *UBE3A–AST* transcript is thought to be responsible for paternal silencing of *UBE3A* expression in *cis* [13]. Because the maternal IC is methylated, the *UBE3A* antisense transcript is not produced from the maternal chromosome, allowing for *UBE3A* expression from the maternal allele (Figure 18.2).

Imprinting defects in AS and PWS most often occur in the absence of IC deletions, and these cases are referred to as primary epimutations [14]. In AS primary epimutation carriers, the paternally imprinted maternal chromosome can be inherited from either the maternal grandfather or the maternal grandmother, suggesting that the defect occurs in the maternal germ line after erasure of imprinting, during establishment or maintenance of the normal maternal imprint. In PWS primary epimutation carriers, the paternal chromosome with a maternal imprint is always inherited from the paternal grandmother, suggesting a failure to erase the grandmaternal imprint in the paternal germ line. This is an example of epigenetic inheritance leading to disease. As of yet there is no mechanistic explanation for the occurrence of these epimutations; however, considering their low frequency, they may occur as a result of chance errors in the normal epigenetic reprogramming that occur in the maternal or paternal germ line.

In summary, AS and PWS are caused by the complex interplay among genetic defects, normal genomic imprinting, and aberrant genomic imprinting or primary epimutations.

Mutations in genes encoding epigenetic regulators

Over 30 genes implicated in ID encode proteins that have a direct role in the epigenetic modification of chromatin structure or are members of core epigenetic regulatory complexes. Here, we discuss ID genes involved in DNA methylation, ATP-dependent chromatin remodeling, and posttranslational modification of histones.

DNA methylation in ID

DNA methylation is an important epigenetic mechanism in the regulation of transcription. Methylation of cytosines (5mC) at CpG sites is generally associated with gene silencing and with condensed heterochromatin. CpG sites are enriched in islands at gene promoters; however, abundant DNA methylation is also seen outside of CpG islands. In addition to 5mC other cytosine modifications have been identified, such as 5-hydroxymethylcytosine (5hmC), which is often found in actively transcribed gene bodies and appears to be enriched in the mammalian brain [15,16]. Mutations in the DNA methylation machinery and in the methyl-DNA-binding protein MeCP2 are implicated in ID, and several studies have shown a role for DNA methylation in the regulation of cognitive function in mammals.

Disruption of DNA methyltransferases in ID

In mammals, there are three active DNA methyltransferases: DNMT1, DNMT3A, and DNMT3B. DNMT3A and DNMT3B are involved in *de novo* DNA methylation, while DNMT1 is involved in maintenance of DNA methylation. DNA methylation was traditionally considered to be a stable epigenetic modification important for development and differentiation. However, it is now known that DNA methylation can undergo a high degree of plasticity in neurons, which may be an important step in the formation of memories [17,18]. Accordingly, inactivation of *DNMT1* and *DNMT3a* in mice and pharmacological inhibition of DNMTs in the rat hippocampus impair long-term memory and long-term synaptic potentiation [18,19]. Mutations in two DNMTs have been linked to neurological

disorders in humans. Dominant mutations in *DNMT1* do not cause ID, but rather are associated with two different neurodegenerative disorders: hereditary sensory neuropathy type IE (MIM 604121) and autosomal dominant cerebellar ataxia, deafness, and narcolepsy (MIM 614116) [20,21]. Recessive mutations in *DNMT3B* cause immunodeficiency-centromeric instability–facial anomalies syndrome 1 (ICF1, MIM 242860), characterized by moderate ID combined with facial characteristics and immune problems [22].

Methyl-CpG-binding protein, MeCP2, and Rett syndrome

MeCP2 was initially discovered as a methylcytosine-binding protein; however, more recent studies established that, in the CNS, MeCP2 binds to both 5mC and 5hmC with high and equivalent affinities [23]. Both 5hmC and MeCP2 are approximately 10 times more abundant in the brain than in peripheral tissues or embryonic stem cells [16,24], suggesting that they embody a CNS-specific epigenetic mechanism of chromatin structure regulation. In humans, mutations in the *MeCP2* gene cause Rett syndrome (RTT, MIM 312750), a female-specific X-linked neurodevelopmental disease [25]. The clinical progression of the disease is unique, with apparently normal early development, followed by regression of growth, motor and social skills, and language beginning 6–18 months after birth. RTT patients exhibit ID, seizures, stereotypic hand movements, and microcephaly, among other deficits.

It has become evident that MeCP2 protein levels as well as various posttranslational modifications greatly influence the outcome on chromatin structure and epigenetic gene regulation. MeCP2 is most abundant in neurons of 6- to 8-week-old mice and displays a widespread genomic distribution suggesting large-scale regulation of chromatin [24]. Fully differentiated neurons contain half as much histone H1 compared with other cell types and MeCP2 is thought to replace one of the histone H1 molecules in these cells (Figure 18.3). This is reinforced by the observation that MeCP2 is able to compete with the histone H1 for chromatin-binding sites *in vitro* and *in vivo*, and that histone H1 protein levels increase twofold in MeCP2-null neurons, possibly as a compensation mechanism [24,26]. The integration of MeCP2 as a variant linker molecule between histone octamers is consistent with the genome-wide localization of the protein, which was detected in ChIP-sequencing experiments [24]. When protein levels are high, MeCP2 appears to act predominantly as a transcriptional activator, likely by a mechanism involving binding to 5hmC-rich gene bodies [23]. This, however, does not preclude a repressive function at specific genomic sites and at pericentromeric heterochromatin through 5mC binding, co-repressor complex recruitment, and/or histone deacetylation [27,28] (Figure 18.3). Despite its global distribution, MeCP2 still participates in the regulation of individual genes. Several activity-responsive genes are directly controlled by MeCP2, which binds to methylated regions of their promoter. Depolarization or synaptic activity can induce calcium-dependent phosphorylation of MeCP2. This can have several outcomes, one being the release of MeCP2 from target genes, such as *BDNF*, resulting in increased transcription [29] (Figure 18.3). These studies underscore the important and complex role of MeCP2 in the epigenetic regulation of brain function.

Many proteins have been identified that bind to MeCP2 including co-repressors (SIN3A, SKI, NCOR1, RCOR1), heterochromatin proteins (HP1, ATRX, cohesin, DNMT1), transcriptional activators (CREB1, YY1, SMARCA4), and RNA-binding proteins (YB1) [30]. Importantly, neuronal-activity-induced phosphorylation of MeCP2 can modify its protein interactions, as was demonstrated for the HDAC-containing nuclear receptor co-repressor (NCOR) complex [31]. Of particular interest in the context of this chapter are the interactions formed by MeCP2 with other proteins involved in

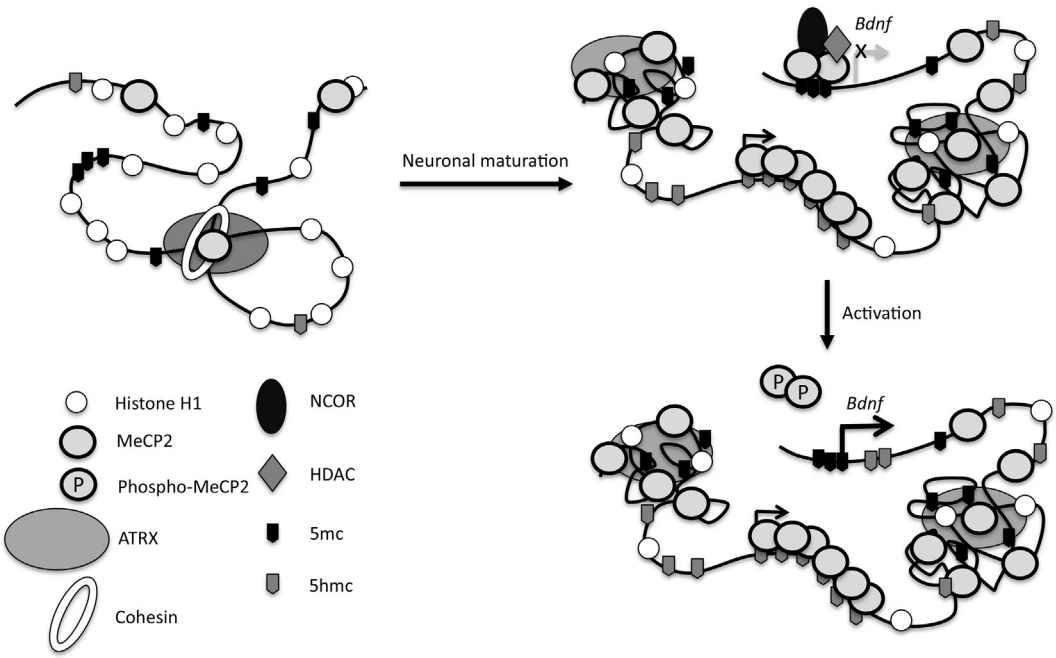

FIGURE 18.3

MeCP2 interacts with various factors to regulate neuronal gene expression. In early stages of brain development, 5hmC and MeCP2 levels are still minimal but can regulate gene expression through their interactions with cohesin and chromatin remodeling proteins such as ATRX. Upon maturation, levels of 5hmC and MeCP2 increase dramatically. MeCP2 acts as a histone H1 linker and regulates chromatin globally. MeCP2 binds to 5hmC located within highly expressed genes and can participate in gene activation. It can also bind to 5mC at gene promoters to repress genes via recruitment of co-repressors such as NCOR and HDACs. Phosphorylation of MeCP2 upon neuronal activation reduces its interaction with methylated promoters allowing expression of genes such as *BDNF*.

syndromic ID. This is illustrated by MeCP2's association with CREB1 at several target promoters [28]. CREB1 is a key participant in learning and memory processes and functions as a transcriptional activator with the ID-linked proteins CBP and p300, causative factors in Rubinstein–Taybi syndrome [32]. SMC1, a subunit of the cohesin complex, binds to MeCP2 in the mouse brain, and has been implicated in cases of the ID disorder Cornelia de Lange syndrome [33,34]. An interaction with the ATRX protein, which is aberrantly expressed in the alpha thalassemia X-linked mental retardation (ATRX) syndrome, was identified by yeast two-hybrid assays and by co-immunoprecipitation in mouse brain tissue [34,35]. Notably, truncating RTT mutations that delete one or more of MeCP2's AT-hook regions and the A140V mutation in MeCP2 that causes ID in males prevent ATRX localization at pericentromeric heterochromatin, suggesting that the binding of ATRX to highly condensed chromatin via MeCP2 is critical for normal human cognition [35,36] (Figure 18.3). These few

examples underscore the extensive interplay between the epigenetic pathways leading to impaired cognition and the importance of investigating causative factors in rare ID disorders.

Disruption of ATP-dependent chromatin remodeling in ID

Chromatin remodeling complexes use energy from ATP hydrolysis to shape the chromatin landscape by moving, removing, destabilizing, and replacing nucleosomes. Chromatin remodeling complexes fall into four different subfamilies: SWI/SNF, INO80/SWR1, ISWI, and CHD [37]. The dynamic regulation of nucleosome position and stability by these complexes is vital for the regulation of gene transcription and other cellular processes such as DNA replication and DNA repair. The catalytic components of chromatin remodeling complexes are members of the SWI/SNF superfamily of proteins with a DEAD/H ATPase/helicase domain. Several chromatin remodeling ATPases and other core members of chromatin remodeling complexes are implicated in ID.

Alpha thalassemia mental retardation syndrome and the ATRX protein

The ATRX protein possesses a DEAD/H ATPase/helicase domain and localizes to highly repetitive and heterochromatic regions of the genome [38]. Mutations in *ATRX* are the principal cause of the ATR-X syndrome (MIM 301040), an X-linked ID disorder that only affects boys [39]. Females are protected from the disease by extensive skewing of X chromosome inactivation resulting in expression of the wild-type copy of ATRX in most cells of the body. Hemizygous males display moderate to severe ID, microcephaly, seizures, myelination defects, atypical facies, and various skeletal abnormalities. Alpha thalassemia and urogenital defects are also frequently observed in affected individuals. The mutations identified thus far in ATR-X patients are predominantly hypomorphic, suggesting that a low level of active protein is required to achieve development to term, albeit with many ensuing abnormalities.

Despite many years of research, the function of ATRX in the epigenetic regulation of gene transcription remains elusive [38].The protein consists of an amino-terminal globular ATRX–DNMT3–DNMT3L (ADD) domain that recognizes and binds histone H3K9me3 (a mark of condensed chromatin) in the context of unmethylated H3K4 [40]. Downstream of the ADD domain is an HP1-binding sequence also predicted to help target ATRX to heterochromatin [41]. Near the C-terminus are seven highly conserved collinear domains that confer ATPase enzymatic activity. A role for ATRX as a transcriptional regulator was suspected due to its chromatin remodeling activities and also based on the observation of reduced alpha-globin expression in ATR-X patients. Microarray analyses of the ATRX-null mouse forebrain reveal that numerous genes exhibit altered transcript levels when ATRX is absent and suggest that ATRX can act as both an activator or a repressor of gene expression [42]. Among the affected genes was a group of imprinted genes that showed increased expression in the ATRX-null brain [34]. A more focused investigation of the *H19/Igf2* domain revealed that ATRX forms a complex with MeCP2 and cohesin at the H19-IC specifically on the maternal allele. Loss of ATRX resulted in decreased binding of cohesin subunits and of the CTCF protein at the maternal H19-IC. Using chromosome conformation capture (3C)-based techniques, we demonstrated that loss of either MeCP2 or ATRX abrogates long-range chromatin interactions formed by the H19-IC *in vivo*. Importantly, loss of either MeCP2 or ATRX results in altered nucleosome positioning on the maternal IC, with nucleosomes blocking the CTCF-binding sites in mutant brains

(our unpublished data). Thus, MeCP2 can recruit ATRX to sites other than pericentromeric heterochromatin in an allele-specific manner. Once in place, ATRX uses its ATP-dependent chromatin remodeling activities to maintain a large linker region required for stable CTCF binding. As predicted from this model, loss of MeCP2 or ATRX prevents CTCF binding at the H19-IC with resulting alterations of long-range chromatin interactions and gene expression. These new findings emphasize the extent of cooperation that exists among various epigenetic factors linked to ID. Of interest is the recent discovery that *CTCF* gene mutations cause ID in humans and that CTCF regulates chromatin interactions and expression of the FXS gene, *FMR1* [43,44]. Additional investigations will be required to ascertain the extent of the relationship between these factors and their concerted effects on gene expression in the developing and mature brain. Nevertheless, the current model reiterates the importance of epigenetic modifications and chromatin structure for the normal acquisition of cognitive abilities.

Mutations in core members of the BAF chromatin remodeling complex cause ID

Recently, mutations were identified in many core members of the SWI/SNF chromatin remodeling complex, BAF, in individuals with diverse forms of ID. Tsurusaki et al. [45] identified dominant *de novo* mutations in *SMARCB1* in two out of five individuals with Coffin–Siris syndrome that were analyzed by exome sequencing. They then sequenced 15 genes encoding BAF complex members in an additional 23 Coffin–Siris individuals. Remarkably, they identified BAF component mutations in 20 out of 23 patients, including six different complex members: *SMARCB1*, *SMARCA4*, *SMARCA2*, *SMARCE1*, *ARID1A*, and *ARID1B* [45]. Despite the success of this study, Coffin–Siris syndrome has since been reconsidered because patients diagnosed with this disorder have such a broad clinical heterogeneity that it does not appear to be a distinct clinical entity [46]. As a result, *ARID1B*, *ARID1A*, *SMARCB1*, and *SMARCA4* mutations are now classified as mental retardation, autosomal dominant 12 (MRD12) (MIM 614562), MRD14 (MIM 614607), MRD15 (MIM 614608), and MRD16 (MIM 614609), respectively. *SMARCE1* mutations are not officially classified as disease causing because only one patient with a mutation in this gene has been identified [45]. *SMARCA2* mutations are now classified as causative for Nicolaides–Baraitser syndrome (MIM 601358) [46,47]. In addition, another member of the BAF complex, ACTB, has been implicated in an ID disorder called Baraitser–Winter syndrome-1 (MIM 243310) [48]. Despite the clinical variability caused by mutations in BAF complex members, it is striking that dominant mutations in so many members of this protein complex are found in ID (Figure 18.4). This underscores the idea that ID, although genetically heterogeneous, is caused by disruption of a limited number of cellular pathways or protein complexes.

It is not clear how disruption of BAF complex members leads to ID. However, several studies have revealed a complex and multifaceted role for the BAF complex in the mammalian nervous system [49]. The BAF complex exists in different forms at different stages of neuronal development, and it is thought that different permutations of the BAF complex help to drive the differentiation of pluripotent stem cells to neural progenitor cells and fully differentiated postmitotic neurons. Interestingly, two BAF complex members, ACTL6A and PHF10, are sufficient to maintain neuronal progenitor proliferation. During exit from the cell cycle and differentiation into postmitotic neurons, these two subunits are replaced by DPF1, DFP3, and ACTL6B and this subunit exchange is essential for neuronal differentiation [50]. The BAF complex present in postmitotic neurons, called nBAF, can control dendrite development by binding to CREST (Ca^{2+}-responsive transactivator) and by regulating genes that are essential for dendritic outgrowth [51]. One of the neuron-specific subunits of

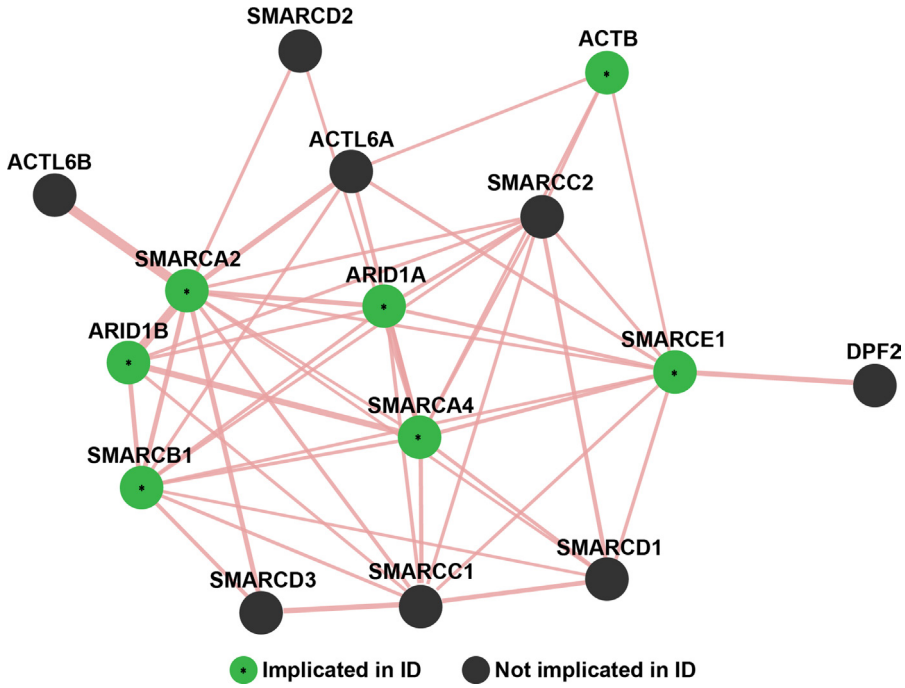

FIGURE 18.4

Several components of the Brg1-associated factor (BAF) complex are disrupted in ID. A protein network of BAF complex members was generated using Genemania (www.genemania.org) and displays annotated direct physical protein interactions. Proteins represented by nodes with an asterisk are implicated in ID.

nBAF is the actin-related protein ACTL6B. Mice with heterozygous *ACTL6B* mutations survive to adulthood and have defects in long-term memory, while maintaining normal short-term memory. The same is also true for mice that overexpress a dominant negative ACTL6B protein in postmitotic neurons. These long-term memory defects can be rescued by introducing wild-type ACTL6B into the adult hippocampus [52]. This demonstrates a role for the nBAF complex in acute processes that are required for memory consolidation in postmitotic neurons. All ID-associated mutations affecting BAF complex proteins are found in subunits that are present in all different forms of the complex.

Mutations in the CHD chromatin remodeling family cause ID, autism, and epilepsy

The CHD family of chromatin remodelers contain N-terminal chromodomains in addition to their DEAD/H ATPase domain. Mutation of *CHD7* is the most common cause of the ID disorder known as CHARGE syndrome (MIM 214800), an acronym for the most prominent clinical features: coloboma, heart anomaly, atresia of the nasal choanal, retardation, and genital and ear anomalies [53]. The CHD family consists of nine proteins that are categorized into subfamilies based on variations in domain structure [37]. These different subfamilies appear to have different roles in transcriptional regulation.

CHD1, for example, is largely associated with maintenance of active chromatin, and CHD2/3 are members of the repressive NuRD (nucleosome remodeling deacetylation) complex [37]. The role for CHD family proteins in the nervous system has not been extensively studied; however, a recent report has implicated CHD7 in adult neurogenesis in mice with targeted knockout of CHD7 in adult neuronal stem cells [54]. CHD7 is required for expression of two key neuronal determination factors, Sox4 and Sox11, in regions of adult neurogenesis. This may in part explain some of the neurological symptoms of CHARGE syndrome, although further investigation is needed into the role of the CHD chromatin remodeling family in cognitive processes. Notably, *de novo* mutations in *CHD2* and *CHD8* were identified in several patients with epileptic encephalopathy [55] and autism spectrum disorder [56–58], respectively, suggesting a wider role for this protein family in neuronal development or function.

SRCAP mutations cause Floating-Harbor syndrome

Recently, heterozygous mutations in *SRCAP* (SNF2-related CBP activator protein) were identified in patients with Floating-Harbor syndrome (MIM 136140), an ID disorder characterized by short stature, delayed bone age, delayed speech development, and typical facial features [59]. SRCAP is a member of the INO80 family of DEAD/H ATPases characterized by a large insertion in the ATPase/helicase domain. SRCAP was originally identified as a binding partner and transcriptional coactivator for the histone acetyltransferase CREBBP (CREB-binding protein) [60]. Interestingly, CREBBP and its paralog p300 are mutated in the ID disorder Rubenstein–Taybi syndrome (discussed below), which shares some common phenotypic features with Floating-Harbor syndrome [32]. Although these studies do link SRCAP with the cAMP signaling pathway, which is a central pathway in learning and memory, the role of SRCAP in the nervous system has not been studied.

Histone methylation in ID

Histone lysine methylation is a key epigenetic mark for the regulation of transcription and is mediated by lysine methyltransferases (KMTs) and lysine demethylases (KDMs). Methylation at various lysine residues within the N-terminal tails of histones can have different effects on gene transcription [61]; for example, H3K4 trimethylation is associated with active transcription, but H3K27 trimethylation is associated with transcriptional repression. Methylated lysine residues can be present in either a mono-, di-, or trimethylated state, and the number of methylation groups at a particular residue can have different outcomes with respect to transcription. For example, H3K9 di- and trimethylation are associated with repressive heterochromatin, while H3K9 monomethylation is found at active enhancers. Several KMTs and KDMs have been implicated in ID.

Disruption of COMPASS-like complexes causes ID

Histone lysine methylation is mediated by proteins containing a SET domain [61]. The *Set1* gene, in yeast, was one of the original SET domain proteins to be described and was shown to be present in a large protein complex named COMPASS (complex of proteins associated with *Set1*). COMPASS complexes mediate H3K4 methylation, a chromatin mark associated with active transcription. In mammals, there are six known H3K4 methyltransferases, which fall into three

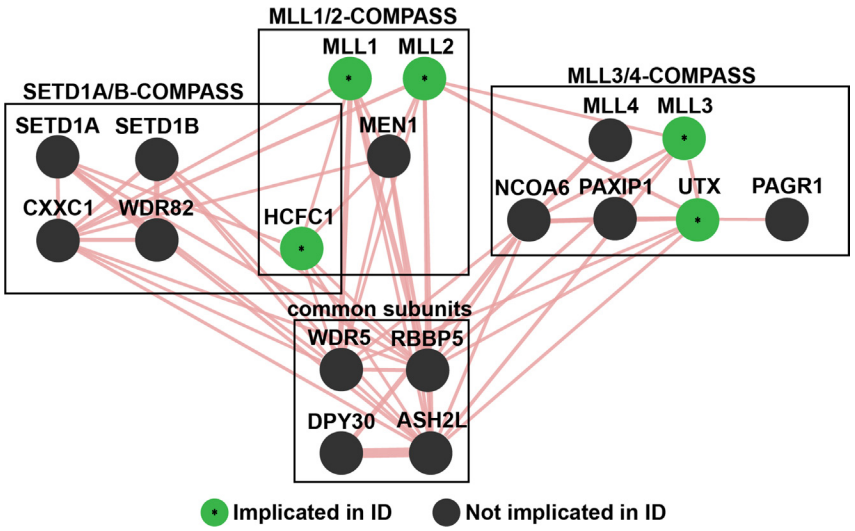

FIGURE 18.5

Several components of COMPASS-like complexes are implicated in ID. A protein network of COMPASS complex members was generated using Genemania (www.genemania.org) and displays annotated direct physical protein interactions. Proteins represented by nodes with asterisk are implicated in ID.

paralogous sets (SETD1A/B, MLL1/2, and MLL3/4). These three groups are present in three distinct mammalian COMPASS-like complexes distinguished by the SET-domain subunits and other complex specific subunits: SETD1A/B–COMPASS, MLL3/4–COMPASS, and MLL1/2–COMPASS (Figure 18.5) [61,62]. All COMPASS complexes contain the core components ASH2L, DPY30, RBBP5, and WDR5. The SETD1A/B and MLL1/2 COMPASS complexes contain HCFC1, which is also found in other histone modification complexes but not in MLL3/4–COMPASS. WDR82 and CXXC1 are specific for SETD1–COMPASS; MEN1 is specific for MLL1/2–COMPASS; and UTX, PAGR1, PAXIP1, and NCOA6 are specific for MLL3/4–COMPASS. SETD1–COMPASS complexes mediate global H3K4 trimethylation in mammalian cells and in *Drosophila*, but the MLL COMPASS complexes have a more limited and specific role in H3K4 methylation [63,64]. The MLL1/2 complex mediates H3K4 trimethylation at *HOX* genes and appears to deposit H3K4me3 at bivalent promoters in mouse embryonic stem cells [65,66]. MLL3/4 is involved in nuclear receptor-mediated transactivation and was recently shown to mediate H3K4me1 at enhancers [62,67].

Several members of the COMPASS complexes are implicated in ID (Figure 18.5). Mutations in *HCFC1* are associated with ID and specific metabolic defects (cobalamin disorder, MIM 309541) [68]. Other COMPASS members involved in ID include MLL2 and the histone demethylase UTX (KDM6A), which are both implicated in Kabuki syndrome (MIM 605130 and 147920) [69,70]. In addition, *MLL1* is the causative gene for Wiedemann–Steiner syndrome (MIM 605130), and an *MLL3* mutation was found in a single patient who was originally diagnosed with Kleefstra syndrome [71,72]. It is notable that ID-causing mutations, to date, are not found in the core COMPASS components or in the SETD1 branch, which regulates global cellular H3K4me3. Rather, mutations

are limited to the MLL1/2 and MLL3/4 branches of the complex that have more subtle effects on gene expression and H3K4 methylation. The presence of ID-causing mutations in the MLL1/2– and MLL3/4–COMPASS complexes suggests a role for these complexes in cognitive function. Interestingly, H3K4me3 was found to be increased in the rat hippocampus after fear conditioning, and *Mll1* heterozygous mice display memory defects in contextual fear conditioning [73]. This suggests that the MLL family may regulate learning and memory through histone methylation of neuronal genes; however, no specific target genes or mechanisms have been described that would explain the role of MLL COMPASS complexes in ID.

Kleefstra syndrome is caused by mutations in EHMT1

The Kleefstra syndrome (MIM 610253) phenotype was originally identified by clinical examination of individuals with a recurrent deletion at chromosome 9q34.3 [74]. The common phenotype associated with these deletions includes severe ID, hypotonia, and recognizable facial features. Further genetic studies revealed that this phenotype is caused by haploinsufficiency in one gene in the 9q34.3 region, the Euchromatin histone methyltransferase 1 (*EHMT1*) gene [75]. EHMT1 and its paralog EHMT2/G9a form a complex that is important for H3K9me2 in euchromatic regions of the genome [76]. In mice, heterozygous loss of *EHMT1* in whole animals and homozygous loss of *EHMT1* in the adult brain causes a range of defects in behavior and learning [77–79]. *EHMT* heterozygous mice also show defects in hippocampal electrophysiology and dendrite morphology [77]. Interestingly, defects in dendrite morphology have also been observed in a *Drosophila EHMT/G9a* mutant [80]. Loss of *Drosophila EHMT/G9a* causes defects in short- and long-term memory and appears to mediate H3K9me2 at many known learning and memory genes [80]. H3K9me2 is also dynamically regulated in response to fear conditioning in different regions of the rat brain, including the hippocampus and entorhinal cortex [73,81]. Thus, there is compelling evidence that EHMT proteins are regulators of cognitive function across evolution. Despite this, the precise mechanism of EHMT proteins in learning and memory has yet to be elucidated.

Disruption of the histone methyltransferases NSD1 and EZH2 causes ID-associated overgrowth disorders

Overgrowth syndromes are a group of disorders characterized by an abnormal increase in size of the whole body or a specific body part. Sotos syndrome (MIM 117550), characterized by pre- and postnatal overgrowth, macrocephaly, advanced bone age, typical facial features, and ID, is caused in part by disruption of the KMT protein NSD1 [82]. *NSD1* mutations were also observed in patients with Beckwith–Wiedeman syndrome (BWS) (MIM 130650), an overgrowth disorder that is typically caused by imprinting defects involving a cluster of genes on chromosome 11p15 [83]. The clinical overlap between Sotos syndrome and BWS led to the proposition that NSD1 is involved in imprinting of the chromosome 11p15 region [83]. Weaver syndrome (MIM 277590) is another overgrowth syndrome that shares a high phenotypic similarity with Sotos syndrome. Interestingly, *NSD1* mutations were identified in patients who were initially diagnosed with Weaver syndrome [84]. Weaver syndrome is also caused by disruption of the KMT protein EZH2 [85]. The observed phenotypic overlap resulting from *NSD1* and *EZH2* mutations suggests that the corresponding proteins act in similar epigenetic regulatory pathways. NSD1 preferentially deposits H3K36me3, a histone mark that is found in gene bodies and promoters of actively transcribed genes. In contrast, EZH2 shows specificity for H3K27me3,

which is associated with transcriptional repression by the polycomb group proteins. It is not clear as to why mutations in these two epigenetic regulators with quite different molecular functions cause such similar phenotypes; however, the answer may lie in the type of mutation. *NSD1* mutations in Sotos syndrome typically cause loss of function, whereas *EZH2* mutations in Weaver syndrome are predicted to give rise to the synthesis of mutant EZH2 protein, with possible gain-of-function effects [86]. Although this hypothesis is not confirmed, it will be interesting to see the effect of *EZH2* mutations on protein function and to further elucidate the role of both of these proteins in neuronal processes.

Disruption of histone demethylases causes ID

In addition to the COMPASS component UTX (KDM6A, involved in Kabuki syndrome [69], discussed above), two other KDMs are implicated in ID: JARID1C (KDM5C) and PHF8. Mutations in *JARID1C* cause syndromic ID with variable severity of phenotypes [87], and mutations in *PHF8* cause ID with cleft lip/palate [88]. KDM6A demethylates H3K27me3, which is antagonistic to the activity of EZH2 [89]. KDM5C specifically acts as a demethylase for di- and trimethylated H3K4me3, thus acting antagonistically to the COMPASS complexes [90]. PHF8 demethylates histone marks such as H3K9me1 and H3K9me2, but not H3K9me3 [91], suggesting an antagonistic activity to the EHMT proteins. Recently, PHF8 was shown to be a regulator of cytoskeletal genes, and it was suggested that the misregulation of these genes leads to defects in neurite outgrowth in a neuroblastoma cell line and in primary neuronal cultures from the mouse cerebral cortex [92]. Aside from this link to neurite outgrowth, the role of histone demethylases in brain function remains unknown.

Histone acetylation in ID

Acetylation of lysine residues on histones H3 and H4 is regulated through a balance between lysine acetyltransferases (KATs) and HDACs. Acetylation can occur at many different lysine residues in the N-terminal tails of histones H3 and H4, and this acetylation is usually associated with active transcription. Many studies suggest that the interplay between KATs and HDACs is critical for long-term memory, and this subject has been reviewed extensively [93]. In general, histone acetylation in the brain favors memory formation; consequently, HDAC inhibitors have been used successfully to enhance memory in model organisms.

Histone acetyltransferases in ID

Mutations in three genes encoding KATs cause ID. Rubinstein–Taybi syndrome is caused by mutations in the *CREBBP* and *P300* genes, encoding paralogous KATs that regulate transcription in concert with CREB. CREBBP is a well-established regulator of long-term memory formation in mice, a subject that has been reviewed extensively [94]. CBP acts downstream of cAMP signaling to regulate the learning-induced activation of gene transcription. KAT6B is the other KAT implicated in ID, and heterozygous mutations were identified in individuals with Genitopatellar syndrome (MIM 606170) and SBBYSS (MIM 603736) [95,96]. In mice, KAT6B is expressed in a subset of adult neuronal stem cells and is thought to contribute to adult neurogenesis and stem cell multipotency by regulating the establishment and self-renewal of these cells [97,98]. Thus, KAT6B and CREBBP/p300 have very

different expression patterns and biological functions, suggesting a differing pathology in the respective human ID disorders.

In addition to mutations affecting KAT proteins, the *KANSL1* gene has recently been implicated in Koolen–deVries syndrome (MIM 610443) [99]. KANSL1 is an essential member of the NSL complex containing the H4K16 acetyltransferase KAT8 [100]. The *KANSL1* gene appears to be required for learning and synaptic development in *Drosophila* [99,101]; however, the underlying mechanisms of the KANSL1 protein and the NSL complex in the brain remain unknown.

Histone deacetylases in ID

Mutations in two HDACs have been shown to cause ID. Mutations in *HDAC8* cause Wilson–Turner syndrome and Cornelia de Lange syndrome [102,103]. Most cases (~60%) of Cornelia de Lange syndrome are caused by disruption of the cohesin-loading protein, nipped-B-like protein (NIPBL), but 5% of cases are caused by missense mutations and small in-frame deletions in the *SMC1A* and *SMC3* cohesin subunits. HDAC8 deacetylates SMC3 at prophase and anaphase upon its release from chromatin, allowing proper recycling and reloading of cohesin on chromatin in telophase by NIPBL. Thus, reduced activity of HDAC8 results in accumulation of acetylated SMC3, altered occupancy of cohesin on chromatin, and abnormal gene expression. In mice, loss of HDAC8 was shown to affect skeletal development in association with the repression of specific genes in cranial neuronal crest cells [104]. This shows that HDAC8 can regulate transcription; however, the epigenetic role of HDAC8 in the brain remains unexplored. HDAC4 mutations cause brachydactyly mental retardation syndrome (MIM 600430) [105]. It is a class II HDAC, and mutation of this gene causes defects in learning and memory and long-term synaptic potentiation [106,107]. HDAC4 associates with neuronal chromatin to repress synaptic genes in the absence of NMDA signaling [106]. NMDA signaling is thought to be activated during learning, causing shuttling of HDAC4 to the cytoplasm and allowing activation of synaptic genes and possibly the strengthening of synaptic connections required for learning and memory. These results contrast with the findings that increasing acetylation in the brain through inhibition of the class I HDAC, HDAC2, leads to better memory formation and increased long-term synaptic potentiation [108]. This paradoxical difference in memory regulation by HDACs may reflect the distinctive enzymatic properties of class I (e.g., HDAC2) and II (e.g., HDAC4) HDACs; however, these mechanisms remain to be eluded.

Histone ubiquitination in ID

Ubiquitination of histones H2A and H2B is involved in epigenetic regulation of transcription. H2A ubiquitination participates in polycomb-mediated gene repression [109], and H2B ubiquitination is associated with activation of transcription and is required for differentiation of multipotent stem cells [110,111]. CUL4B and ASXL1 regulate the ubiquitination of histone H2A and are components of different polycomb complexes. *CUL4B* is mutated in a non-specific form of X-linked ID (MRXS15) (MIM 300354), and *ASXL1* mutations were identified in Bohring–Opitz syndrome (MIM 605039) [112,113]. CUL4B represses gene transcription through its association with polycomb repressive complex 2 (PRC2) and monoubiquitination of H2A119 [114]. ASXL1 is a subunit of the polycomb repressive deubiquitination (PR-DUB) complex, which is also involved in gene repression [115]. In

opposition to CUL4B, ASXL1 is required for removal of H2A119 ubiquitination. It is at this point not known why enzymes responsible for both removal and addition of ubiquitin to histone H2A are required for polycomb-mediated gene silencing [116]. Finally, UBE2A, which associates with the E3 ligase BRE1 and catalyzes the monoubiquitination of histone H2B (H2BK120ub1), was shown to be involved in a non-specific form of syndromic ID (MRSX30) (MIM 300860) [110,117]. Although histone ubiquitination appears to play a complex role in epigenetic regulation of gene transcription, its role in the nervous system remains unexplored.

Summary and conclusions

Epigenetic regulation of chromatin structure is emerging as an important mechanism in the etiology of ID. It is clear that aberrant epigenetic silencing of important neuronal genes can contribute to ID, and prominent examples of this include *FMR1* silencing in FXS and UBE3A silencing in AS (Figures 18.1 and 18.2). In addition, many epigenetic regulators appear themselves to be important in the regulation of neuronal processes, and mutations in these genes can also result in ID. Interestingly, many of the mutated genes discussed here encode proteins that work together in common pathways or complexes (Figures 18.3–18.5). This underscores the idea that ID, although genetically very diverse, is caused by disruption of a limited number of cellular processes or pathways [1]. This raises the possibility that a number of ID disorders with a distinct genetic etiology may be treatable by targeting the commonly disrupted mechanism. In this sense, ID should no longer be viewed as a collection of rare disorders but as a single relatively common disorder, with an inherent phenotypic diversity. Indeed, the lines between different ID disorders are becoming increasingly blurred, and there is also a great deal of genetic overlap between ID and other psychiatric disorders such as autism and schizophrenia. Continued research into the (epi)genetic causes of ID and detailed analysis of the underlying protein networks in the nervous system will be essential in moving forward toward therapy. The prospect of targeting epigenetic mechanisms for therapy does exist; however, more research is clearly required to further understand the complexities of epigenetic regulation in the brain.

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Glossary

ATP-dependent chromatin remodeling The movement or ejection or restructuring of nucleosomes requiring energy from the hydrolysis of adenosine triphosphate (ATP).

Euchromatin The less dense form of chromatin that is rich in genes and is thought to be more suitable for active transcription than highly condensed heterochromatin.

Genomic imprinting An epigenetic phenomenon by which certain genes can be expressed in a parent-of-origin-specific manner.

Heterochromatin Dense chromatin that is thought to be less transcriptionally active than euchromatin. Heterochromatin can be defined in two subtypes. Constitutive heterochromatin includes structural regions of the chromosome, such as centromeres and telomeres, whereas facultative heterochromatin is involved in silencing of specific genes in a given cell type.

Imprinting center A chromosomal region that is required for genomic imprinting of a given imprinted region. If the imprinting center is missing or altered, genes in that region will not undergo normal imprinting.

Intellectual disability A disability characterized by significant limitations both in intellectual functioning (reasoning, learning, problem solving) and in adaptive behavior, which covers a range of everyday social and practical skills. This disability originates before the age of 18.

Primary epimutation A heritable change in gene expression that is not associated with any change to the DNA sequence.

Uniparental disomy Inheritance of two copies of a chromosome, or of part of a chromosome, from one parent and no copies from the other parent.

CHAPTER

Epigenetics and Pervasive Developmental Disorders

19

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KEY CONCEPTS

- Despite differing genetics and clinical presentations, neurodevelopmental disabilities (NDDs) share common pathways.
- The common pathways may help us understand how genetics and epigenetics may modify phenotypes.
- Common themes in the NDDs include pathways that impact synapses, and some have coined the term synaptopathies to indicate the disruption of synaptic structure and function.
- Genetic heterogeneity in autism spectrum disorder (ASD) results in genetic risk conferred by multiple loci. With the advent and increasing use of exome sequencing and whole genome oligonucleotide-based microarrays, copy number variations (CNVs) associated with ASD have been uncovered.
- Epigenetics, which refers to changes in gene expression without changes in the base pairs, helps to explain the epidemiology of illnesses such as autism and other NDDs.
- How epigenetic pathways can regulate neuronal differentiation and maturation may reveal novel treatments.

ABBREVIATIONS

aCGH Array comparative genomic hybridization
ADHD Attention deficit hyperactivity disorder
AGRE Autism Genetic Resource Exchange Consortium
AKT Atypical kinase
ALC Acetyl-L-carnitine
AMPA α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AS Angelman syndrome
ASD Autism spectrum disorder
ATRX Alpha thalassemia X-linked mental retardation
AXAS ASD, XLID, ADHD, SZ
BDNF Brain-derived neurotrophic factor
CBP CREB-binding protein
CDKL5 Cyclin-dependent kinase-like 5
CGG Cytosine–guanine–guanine
CNV Copy number variation
COPG2 Coatomer protein complex, subunit gamma 2
CpG Cytosine–phosphate–guanine
CREB cAMP response element-binding protein
DNMT DNA methyltransferase
ELN Elastin
FISH Fluorescence *in situ* hybridization
FMR1 Fragile X mental retardation gene 1
FMRP Fragile X mental retardation protein
FXS Fragile X syndrome
GABR $GABA_A$ receptor subunit
HAT Histone acetyltransferase
HDAC Histone deacetylase
ID Intellectual disability
IMGSAC International Molecular Genetic Study of Autism Consortium
LIMK1 Lim kinase 1
MAPK Mitogen-activated protein kinase
MeCP2 Methyl-CpG-binding protein 2
MEST Mesoderm-specific transcript
mGluR Metabotropic glutamate receptor
mTOR Mammalian target of rapamycin
NDDs Neurodevelopmental disabilities

NDMA *N*-methyl-D-aspartate
NGS Next-generation sequencing
PI3K Phosphatidylinositol 3-kinase
PPI Protein–protein interaction
PWS Prader–Willi syndrome
RT–PCR Reverse transcription–polymerase chain reaction
RTT Rett syndrome
SMS Smith–Magenis syndrome
SNP Single nucleotide polymorphism
SNVs Single nucleotide variants
SWI/SNF SWItch/Sucrose NonFermentable
SZ Schizophrenia
UBE3A Ubiquitin protein ligase E3A
UPD Uniparental disomy
VCFS Velocardiofacial syndrome
VPA Valproic acid
WES Whole exome sequencing
WGS Whole genome sequencing
WS Williams syndrome
XLID X-linked intellectual disability

Introduction

Autism spectrum disorders and neurodevelopmental disabilities

Autism is a neurodevelopmental disability (NDD) defined by the triad of abnormal (or impairment of) social interaction, impairment of communication skills, and stereotypic and repetitive behaviors [1,2]. Many autism spectrum disorders (ASDs) have a strong genetic component and/or a high heritability as suggested by a multitude of family and twin studies; however, in the majority of cases, a genetic basis of ASD remains undiscovered [3]. Phenotypic and genetic heterogeneity contribute to difficulty in finding a genetic basis in many cases. Autism, as originally proposed by Kanner, was thought to be due to an inborn disorder. Folstein and Rutter, however, were the first to demonstrate a greater than 50% concordance for monozygotic versus dizygotic twins, and this is borne out in more recent twin studies observing as high as 90% trait heritability for monozygotic twins [4]. Additionally, family studies suggest an increased risk over the general population for first-degree relatives [5]. The most recent CDC prevalence estimate for ASD is 1/88 [6]. Taken together, these studies indicate a high genetic liability, but they also suggest that there are other non-inherited and environmental factors that may influence the penetrance or expressivity of these disorders with respect to genetic risk factors.

Although ASD and other NDD are known to be highly heritable, the identification of candidate genes causing autism and NDD endophenotypes has been very slow, in part due to clinical heterogeneity of the symptoms and insufficient numbers of participants. The establishment of collaborative groups, such as the Autism Genome Project Consortium and the International Molecular Genetic Study of Autism Consortium (IMGSAC) [7], along with the Autism Genetic Resource Exchange Consortium (AGRE), a repository for genetic information and pedigrees, has been extremely helpful in moving gene identification studies forward [8].

Genetic heterogeneity in ASD results in genetic risk conferred by multiple loci. With the advent and increasing use of exome sequencing and whole genome oligonucleotide-based microarrays, copy number variations (CNVs) associated with ASD have been uncovered [9]. As a result, numerous rare *de novo* and/or inherited CNVs have been implicated in ASD. There is evidence that *de novo* CNVs are more enriched in children with NDDs and ASD than controls [9,10]. In the process, a number of single nucleotide variants (SNVs) have been discovered. The significance and interpretation of these variants remained largely stalled until the arrival of next-generation sequencing (NGS)

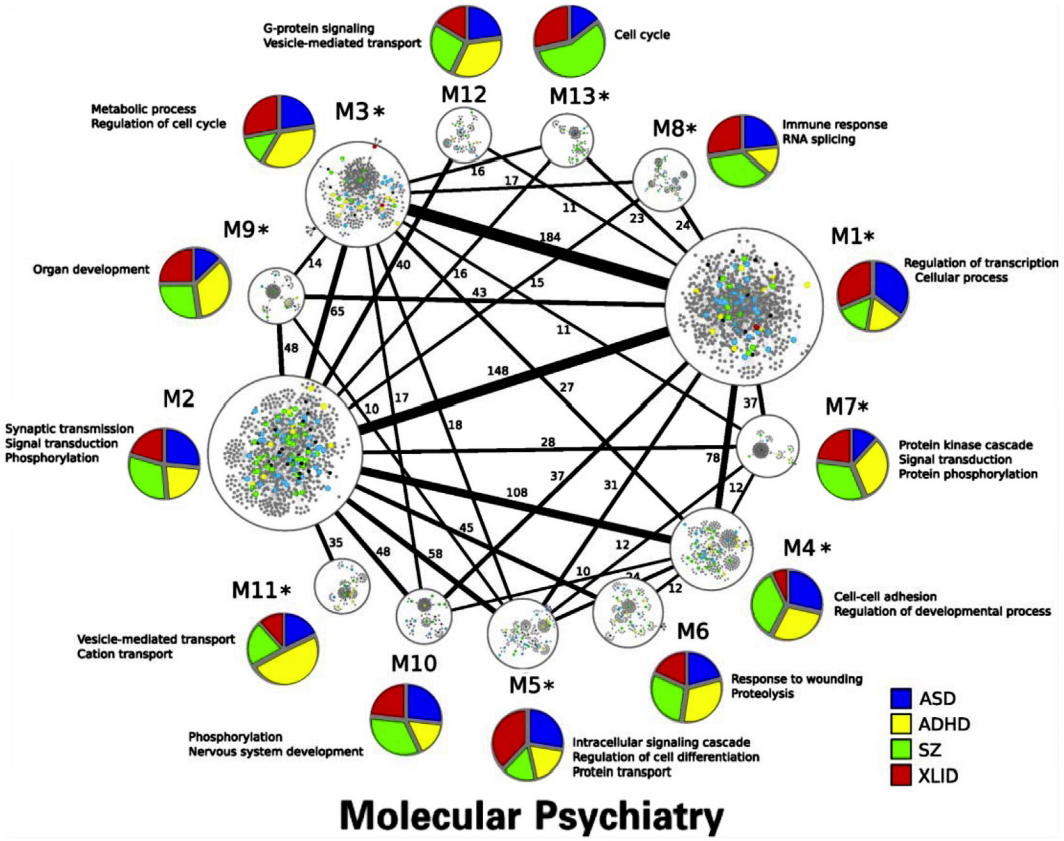

FIGURE 19.1

Graphical representation of the 13 most populated protein modules of the AXAS (ASD, XLID, ADHD, SZ)–PPI (protein–protein interaction) network. The size of each module is proportional to the number of proteins. Weighted lines with numbers represent protein interactions between modules.

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technologies and genomic sequence enrichment methods, such as whole genome sequencing (WGS) and whole exome sequencing (WES). As a result, *de novo* SNVs have also recently been implicated in ASDs through WES involving trios (parent and proband) investigations [11–13]. Taken together, these findings lend support to the hypothesis of the occurrence of *de novo* mutations underlying the genetic basis of many of the patients with ASD and NDD and account for the high prevalence of these diseases, although we are still a long way from understanding the significance of certain of these variants. Due to phenotypic and genotypic heterogeneity, the concept of defining ASD by endophenotypes, or clinical markers that span the spectrum between disease and genotype [14], has led to increased statistical power. For example, in ASD, endophenotypes such as the presence or lack of language, age at first word, gender, presence and severity of repetitive behaviors, and best estimate full scale IQ have been used in an attempt to increase power [15–17]. (See Figure 19.1.)

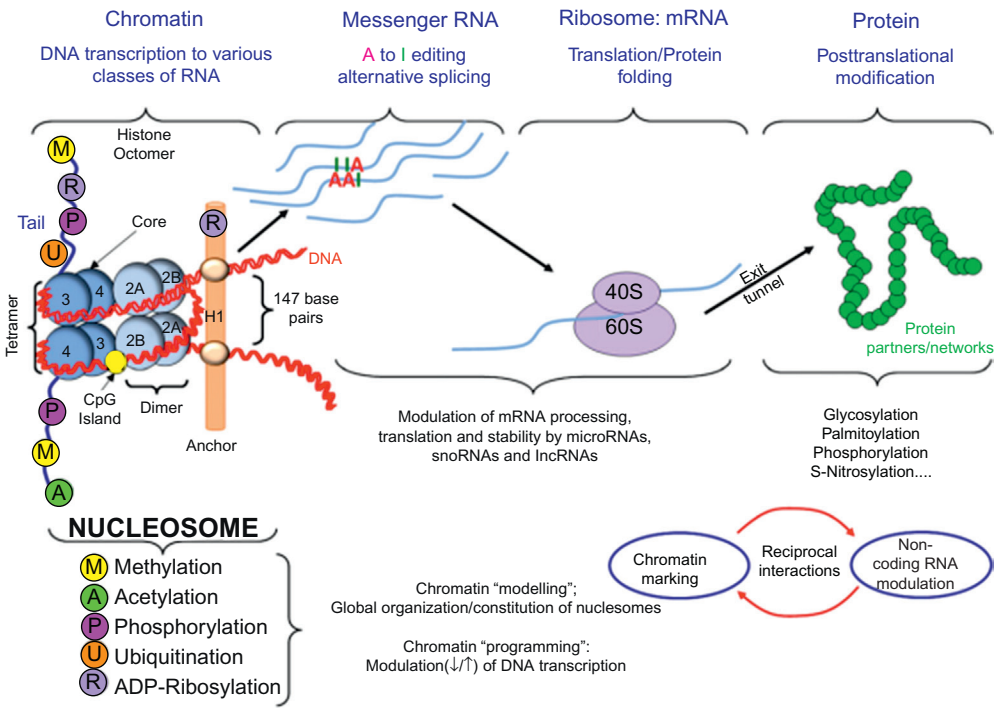

FIGURE 19.2

Epigenetic mechanisms from gene to protein.

Common pathways and mechanisms in NDDs

Despite differing genetics and clinical presentations, the NDDs share common pathways [18]. The common pathways may help us understand how genetics and epigenetics may modify phenotypes. Common themes in the NDDs include pathways that impact synapses, and some have coined the term synaptopathies to indicate the disruption of synaptic structure and function, as exemplified by fragile X syndrome (FXS), Phelan–McDermid syndrome, and Rett syndrome (RTT) [19–22]. A second, but not mutually exclusive, pathway involves disruption of signaling via the Ras–Raf–Kinase cascade. This ultimately impacts mammalian target of rapamycin (mTOR) signaling [23,24], and NDDs following this pathway have been termed rasopathies. (See Figure 19.2.)

Epigenetics as an explanation of variability in developmental disorders

Epigenetics, which refers to changes in gene expression without changes in the base pairs, helps to explain the epidemiology of illnesses such as autism and other NDDs. The complexities of such disorders arise from their etiologic heterogeneity as well as the interacting influence of environmental and genetic factors. Although many developmental disorders and autism may have a genetic basis, recent research demonstrates that an additional interaction between genetic predisposition and

environmental exposures may additionally promote or inhibit gene expression. Environmental effects on genes may occur *in utero* or later in life. An understanding of epigenetics may also help predict individual differences to medication response and is beginning to form the basis for medication trials for neurodevelopmental disorders.

Involvement of epigenetic regulatory mechanisms in the pathogenesis of ASD has been suggested due to the recognition of occurrence of ASD in patients with neurogenetic conditions that arise from disorders involving epigenetic regulatory factors. These conditions include FXS, RTT, and Rubinstein–Taybi syndrome. Common causes of intellectual disability (ID) and autism include chromosome abnormalities, microdeletions, FXS, other X-linked syndromes, and single gene disorders including inborn errors of metabolism, Rubinstein–Taybi syndrome, and RTT. Despite differing genes, these disorders share common defects in neuronal morphology, brain plasticity, and synaptic connectivity often involving changes in dendritic spine morphology and branching. The time of onset and clinical presentation of these developmental disorders suggest overlapping genes in common pathways [25].

Control of transcription is key in the process of gene expression and is the first line of epigenetic control

The control of eukaryotic gene transcription is an important process that involves the precise positioning of histones and initiation factors in time and space. Effects of this determine whether the genome is subject to open or condensed chromatin, governing transcriptional activity, and gene expression. Equally important are mechanisms for recombination, replication, and repair.

Epigenetic mechanisms and intellectual and neurodevelopmental disabilities

The most common epigenetic effects studied in ASD are DNA methylation and histone acetylation. Both of these mechanisms act in a manner to control heritable states of gene expression. Both DNA methylation and histone acetylation and deacetylation are highly dynamic and may be both tissue specific and developmentally regulated. This contrasts with heritable changes in the DNA sequence which are strongly conserved and stable. (See Figure 19.3.)

Histone modification

Histone acetylation opens the chromatin structure and favors gene transcription (i.e., turning the gene on), whereas DNA methylation can cause condensation of the chromatin with resultant gene silencing. Histone deacetylation results in gene silencing [26]. DNA is condensed and packed in the nucleus of eukaryotic cells and wrapped around an octamer of proteins, termed histones. Each octomer is comprised of two copies of each of the core histone proteins: H2A, H2B, H3, and H4.21. Several types of dynamic and reversible posttranslational modifications in histones can occur. These include methylation of lysines and arginines, acetylation, phosphorylation, ubiquitination, ADP ribosylation, and SUMOylation [27,28]. Eukaryotic DNA is compacted into chromatin, which is made up of nucleosome repeats [29]. The nucleosome consists of a core particle composed of a histone octamer that is associated with DNA, as well as a linker DNA that connects all the core particles bound by one H1 linker histone. The histone octamer complex in turn consists of two copies of each of the four histones: H2A, H2B, H3, and H4. Histone H1 or linker histone seals two rounds of DNA

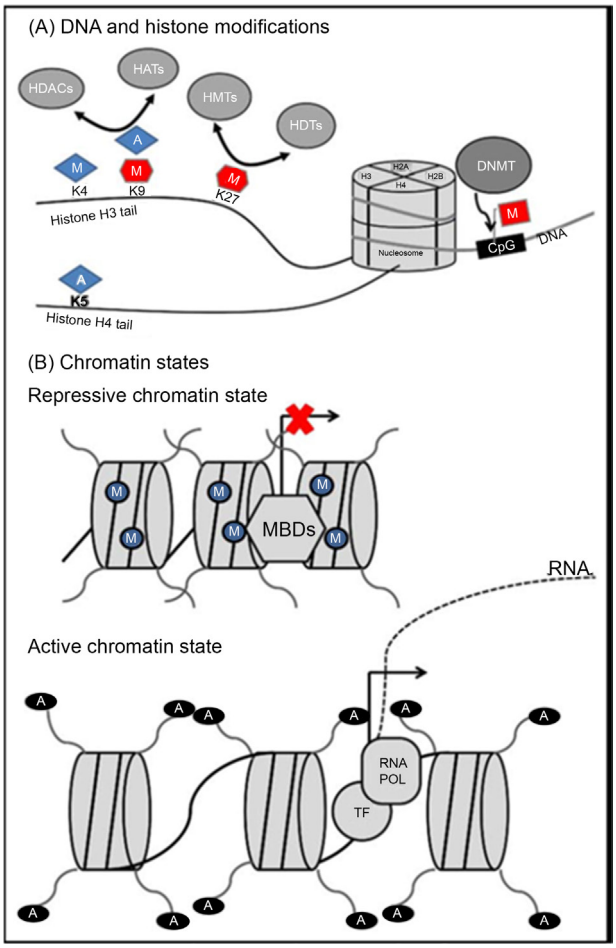

FIGURE 19.3

DNA and histone modification.

at its entry/exit site on the surface of the nucleosome core and thereby stabilizes higher-order chromatin structure [30,31]. Histone H1 has many variants with specificity observed among species, tissue types, and even developmental stage. Histones can be subjected to a variety of covalent, but dynamic, posttranslational modifications including acetylation, phosphorylation, and methylation. All of these mechanisms will determine their interactions with other proteins, serve to regulate chromatin structure, and play a role in remodeling. Histone modification is another epigenetic mechanism that can result in gene silencing. Acetylation of conserved lysine residues present in histone tails is linked to transcriptional activity. Histone acetylation is a hallmark of transcriptionally active regions, whereas hypoacetylated histones are associated with transcriptionally inactive euchromatin or heterochromatin

regions. The equilibrium of histone acetylation/deacetylation is governed by the opposite actions of two sets of enzymes that play an antagonistic role, either attaching or removing the corresponding chemical group in a site-specific manner. The most commonly studied modification of histones involves acetylation, carried out by histone acetyltransferases (HATs) and histone deacetylases (HDACs) which remove acetylation. HATs promote gene expression; HDACs inhibit gene expression. The changes in the posttranslational chemical modifications of histones can also be used as targets for therapeutic intervention. In addition to acetylation or hypoacetylation, there is an additional control with the fact that different degrees of methylation could be correlated with distinct levels of gene silencing. Trimethylation of lysine 9 of histone 3 has been shown to be associated with constitutive heterochromatin. Mono- and dimethylation of the same position are associated with facultative heterochromatin [27]. On the other hand, methylation of lysine 4 of histone H3 is a signal for transcriptional activity and is found at the promoters of active genes [28].

DNA methylation

DNA methylation is the most widely studied epigenetic mechanism and involves covalent attachment of a methyl group (CH_3) at the 5′ position of cytosine residues. Methylation of cytosine forms CpG dinucleotides (the "p" in the CpG notation refers to the phosphodiester bond located between the cytosine and the guanine). These CpG dinucleotides are concentrated in regions known as CpG islands, which are found in promoter regions of genes. Methylation leads to gene silencing [32]. Methylation of DNA plays a role in several biological processes that will be discussed in this chapter. These include parental chromosome imprinting, development, and X-chromosome inactivation [32–34]. The methylation of DNA is processed by a group of enzymes called DNA methyltransferases (DNMTs). The mammalian DNMT family consists of five proteins (DNMT1, 2, 3A, 3B, 3L). DNMT1 is involved in maintenance of the DNA methylation pattern during replication. DNMT3A and DNMT3B are *de novo* methyltransferases. DNMT3L is required to establish maternal genomic imprints during the development of oocytes. Methylation leads to silencing of imprinted genes (in which only one allele is expressed, either from the mother or father). This mechanism is the origin of most cases of Prader–Willi syndrome (PWS) and Angelman syndromes (AS). Methylation also plays a role in X-chromosome inactivation in females and, in accordance with the Lyon hypothesis, serves as a mechanism for dosage compensation (i.e., males have only one X chromosome but generally have the same level of X-derived gene products as females who have two X chromosomes). When X-chromosome inactivation is skewed, it can lead to expression of sex-linked conditions in females, such as ornithine transcarbamylase deficiency, a metabolic disorder associated with cognitive impairment related to hyperammonemic crises.

How do epigenetic modifications contribute to diversity?

The combination of genome modifications allows individual cells to show varying outputs in response to extracellular signaling. For example, the previously mentioned modifications of methylation and histone acetylation and deacetylation allow for complexity in terms of the degree of methylation (mono-, di-, or trimethylation) and protein expression. Furthermore, combinations or sequential acetylation or deacetylation can affect chromatin organization and expression of the corresponding target genes [35]. (See Table 19.1.)

Table 19.1 Examples of Neurodevelopmental Disorders Involving Epigenetics

Disorder	Epigenetic Mechanism	Genetic Mechanism	DNA Methylation Status	Histone Alteration
Fragile X	DNA methylation alteration	Expanded CGG repeats cause gene silencing	Hypermethylation	—
Rett syndrome	DNA methylation alteration	Loss of activity of MeCP2	Hypermethylation	—
Coffin–Lowry	Histone alteration	Impairment of MAPK signaling	—	Hypophosphorylation
Rubenstein–Taybi	Histone alteration/ DNA methylation	CREB regulation	Hypertrimethylation	Hypoacetylation

Examples of human neurodevelopmental disorders with autistic features linking epigenetics and phenotype variation

Rett syndrome/MeCP2 (RTT)

An example of a link between epigenetics and a human developmental disorder on the autism spectrum is the X-linked MeCP2 gene. Affected patients have mutations in the gene encoding the methyl-CpG-binding protein 2 (MeCP2) that attaches to methylated DNA [36,37]. The majority of cases of RTT are the result of *de novo* mutations in this gene that can arise in either parent, but more frequently occur in the paternal gamete. Mutations in MeCP2 cause RTT, a postnatal neurodevelopmental disorder that occurs in 1:8000–1:15,000 female births. It is the second most common cause of ID in females, although it can also affect males who have severe encephalopathy and early lethality. Mutations in the MeCP2 gene account for half of girls who manifest classical RTT. In classical RTT, the girls are apparently normal at birth. There is then a postnatal deceleration of head growth along with neurodegeneration, specifically a loss of purposeful hand skills which are replaced by stereotypical hand wringing, ataxia, cognitive impairment, and social withdrawal. After this initial regression, the second phase of the disorder shows symptom stabilization with improved social contact and eye gaze but gradually leading to slowing of motor functions and further deterioration. Clinical features of individual RTT patients vary significantly among individuals along a spectrum from mild to severe.

Although mutations in MeCP2 were discovered several decades ago, the molecular function of the gene is not fully understood. MeCP2 was originally found to bind methylated DNA and interact with repressor complexes to inhibit and silence its genomic targets, but more recent studies have challenged this observation and suggest a greater role and variability in gene expression. MeCP2 may act as either an activator or a repressor depending on its interacting protein partners and target genes

Another concept is the fact that although RTT is a progressive postnatal onset condition, the consequences of MeCP2 mutation may begin before birth. Several functional characteristics of MeCP2 suggest that it plays a role in synaptic development and function, including its neuronal expression pattern, neuronal activity-dependent regulation of brain-derived neurotrophic factor (BDNF) induction by hippocampal kindling, and developmental expression pattern coinciding with neuronal maturation [38]. A few male cases with RTT who have survived infancy have been reported, but these were

generally mosaic for MeCP2 mutations [39], as germ line MeCP2 mutations lead to a severe neonatal encephalopathy with demise. Recently, a novel X-linked ID syndrome has also been associated with duplications of the MeCP2 chromosomal region [39]. This suggests that careful regulation of the MeCP2 gene is required for typical brain development and function, as both overexpression and reduced expression are associated with ID [40].

What is the function of MeCP2?

MeCP2 is a nuclear protein that colocalizes with methylated heterochromatin in mouse cells. The differential expression of Mecp2/MeCP2 transcripts can be subjected to tissue- and developmental stage-specific regulation. In the brain, differential transcript expression patterns for the two isoforms have been detected [37,38]. The transcript levels are high during embryogenesis and decrease postnatally with brain maturation, but again increase in early adulthood [41]. The MeCP2 gene encodes a nuclear protein whose role is that of a transcriptional repressor, i.e. silences other genes [42]. Mutations in CDKL5 (which encodes cyclin-dependent kinase-like 5) have also been implicated in a RTT-like phenotype [43,44]. MeCP2 and CDKL5 interact both *in vivo* and *in vitro*, suggesting that they belong to a common molecular pathway [45]. This is a common theme in epigenetic modulation [46].

Alpha thalassemia/mental retardation syndrome X-linked

Alpha thalassemia X-linked mental retardation (ATRX), a chromatin-remodeling factor, is another DNA methylation modifier that leads to an X-linked disorder if there is a mutation. Mutations in this gene cause the alpha thalassemia/mental retardation syndrome, which is manifest by intellectual delays, facial abnormalities, urogenital defects, and alpha thalassemia [47]. The ATRX protein is a member of the Switch 2, sucrose non-fermenting 2 (SWI2/SNF2) family of helicases/ATPases that exhibits chromatin remodeling activity [48–50]. Identical ATRX mutations in different patients can result in variable hematological and neurological phenotypes [51]. The ATRX gene, like MeCP2, is X-linked and has been found to require MeCP2 for targeting to heterochromatin. Mutations in ATRX have been found to lead to DNA methylation changes at repetitive elements such as ribosomal DNA, subtelomeric repeats, and Y-specific satellites [52]. (See Figure 19.4.)

Rubinstein–Taybi syndrome

Rubinstein–Taybi syndrome is associated with the dysfunction of a histone acetyltransferase. An autosomal dominant disorder, it is characterized by ID and physical anomalies, most notably broad radially deviated thumbs and phalluses and a postnatal growth deficiency that is later followed by excessive weight gain [53,54]. There are also characteristic dysmorphic facial features and an increased risk for developing tumors [55,56]. Rubinstein–Taybi syndrome is caused by mutations in the cAMP response element-binding (CREB) protein (CBP) gene. As a coactivator of transcription, CBP has dual functions in mediating both gene activation and epigenetic modification. CBP recruits other transcription factors to the transcriptional machinery and demonstrates HAT activity. This has the potential to affect regulation of other genes, but the underlying mechanism linking this epigenetic regulation to brain development is currently unknown.

Coffin–Lowry syndrome

Coffin–Lowry syndrome is a neurological disease caused by a deficiency in a histone phosphorylase. An X-linked disorder, this syndrome is associated with severe ID and a dysmorphic facial

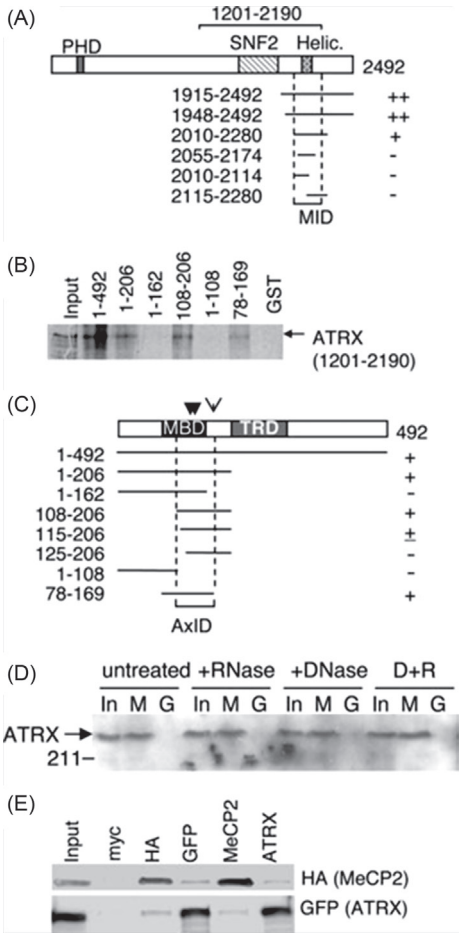

FIGURE 19.4

MeCP2 interacts with ATRX. Yeast two-hybrid assays identified a region of ATRX that interacts with MeCP2.

Reprinted with permission from Nan X, et al. Interaction between chromatin proteins MECP2 and ATRX is disrupted by mutations that cause inherited mental retardation. Proc Natl Acad Sci USA 2007; 104: 2709–14.

appearance. The most salient features include a prominent forehead, down-slanting palpebral fissures, orbital hypertelorism, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Coffin–Lowry syndrome is caused by loss-of-function mutations in the RSK2 gene (also known as RPS6KA3) on chromosome Xp22.2 [57]. RSK2 is a growth factor-regulated serine–threonine protein kinase that acts in the Ras/mitogen-activated protein kinase (MAPK) signaling pathway. Coffin–Lowry syndrome is considered an epigenetic disorder because RSK2 affects chromatin structure through direct phosphorylation of histones as well as by interactions with CREB protein P [58,59].

Epigenetics and imprinting disorders

Imprinting is an epigenetic modification in which the origin of parental chromosomes in the gamete or zygote may lead to differential expression of the two alleles of a gene [60,61]. Maternal and paternal genomes are both required for normal embryonic and postnatal development. Methylation at CpG islands is associated with imprinting, playing an important role in silencing certain genes and activating others. Initial parental-origin imprinting signals are established during gametogenesis. In the preimplantation embryo, the methylation patterns of the gametes are erased, resulting in a decrease in the total level of methylated DNA. After implantation, *de novo* methylation ensues and thereby establishes a new pattern, in which the majority of CpG islands are methylated in a tissue-specific manner. Genomic imprinting is an epigenetic phenomenon in which the activity of the gene is modified depending on the sex of the transmitting parent. Typically, mammals inherit two sets of chromosomes, one from the mother and the other from the father. Most autosomal genes are expressed in both maternal and paternal alleles. However, imprinted genes show expression from only one allele (the other is silenced), and this is determined during production of the gamete. Imprinting implies that the gene carries a "tag" placed on it during spermatogenesis or oogenesis. Imprinted genes are important in development and differentiation, and if expression from both alleles is not maintained then disturbances in development can result [38]. A genome-wide search of imprinted human genes has identified more than 150 candidates. The first human imprinting disorder discovered was PWS, which is caused by a paternal deletion in chromosome 15 or maternal uniparental disomy (UPD) (in which both chromosome 15s come from the mother). Other examples of imprinted neurogenetic disorders include AS and Beckwith–Wiedemann syndrome.

Prader–Willi syndrome

Prader–Willi syndrome (PWS) is characterized by severe hypotonia and feeding difficulties in infancy, followed by development of an insatiable appetite and obesity by school age. PWS features significant motor and language delays in the first two years of life, borderline to moderate ID, and severe behavioral problems, including compulsive and hording behaviors. Many affected children manifest the core criteria for the diagnosis of autism. Hypogonadism manifests as genital hypoplasia, incomplete pubertal development, and infertility in both males and females [62].

The estimated prevalence of PWS is 1:10,000–1:22,000 [63] and the genetic defect involves one of three mechanisms: deletion, imprinting defect, or maternal UPD [40]. Approximately 75% of patients with PWS have a specific microdeletion, 15q11.2–q13. This can be detected by high-resolution chromosomal analysis (i.e., greater than 550-band level), by fluorescence *in situ* hybridization (FISH) testing, or by array comparative genomic hybridization (aCGH). One percent of patients with PWS have a detectable chromosomal rearrangement resulting in a deletion of 15q11.2–q13. Less than 1% of patients have a balanced chromosomal rearrangement with breakpoints within 15q11.2–q13. Less than 5% have an imprinting defect, and ~20% have UPD [64].

Angelman syndrome

Although the same chromosome region is involved, a vastly different phenotype compared with PWS is the Angelman syndrome (AS). Maternal deletion of the region 15q11–q13, and specifically involving the UBE3A gene, causes AS. Paternal genes in this region are silenced. AS has a characteristic facial appearance (hypopigmented skin and eyes, prominent mandible, tongue thrusting, wide mouth, wide-spaced teeth), developmental delay, progressive microcephaly, ataxic gait, absence of speech, seizures,

Table 19.2 Genetic Mechanisms Leading to Angelman Syndrome

Type of Mutation	Mechanism	Percentage	Comments
Class I	Deletion of chromosome 15q11–q13; includes chromosome rearrangements	70–75%	Most deletions are *de novo* and of maternal origin. Microdeletions occur due to unequal crossing over between low copy repeats (duplicons) within the chromosome region. The duplicons contain the *HERC2* gene, a highly conserved gene which when mutated leads to developmental and fertility problems
Class II	Uniparental disomy for chromosome 15 and failure to inherit a maternal copy of UBE3A	2–3%	—
Class III	Abnormal methylation, consistent with a defect in imprinting	2%	—
Class IV	UBE3A mutations (ubiquitin protein ligase targets proteins for degradation)	5–10%	—
Class V	No genetic abnormality identified	12–15%	—

and inappropriate bouts of laughter [65]. The prevalence of AS is about 1:20,000. There are four genetic mechanisms that can cause AS, and a classification scheme has been developed to group patients according to etiology. The basic underlying defect involves the UBE3A gene, which encodes ubiquitin protein ligase E3A. This enzyme is involved in targeting proteins for degradation within cells. Both copies of the UBE3A gene are active in most of the body's tissues, but in the brain only the maternal copy is normally active. If this copy is mutated or lost, it affects many developmental processes. (See Table 19.2.)

Genotype–phenotype correlations have been shown for AS. Patients with chromosome 15 deletions are most severely affected with a higher incidence of epilepsy, microcephaly, ID, absence of speech, and hypopigmentation [66]. Patients with UPD tend to have a lower incidence of seizures, microcephaly, and hypopigmentation. They may have sparse speech, normal growth parameters, and less obvious dysmorphisms. Patients with imprinting defects are less likely to have seizures, microcephaly, and hypopigmentation, and they show better motor and communication skills. Individuals with UBE3A mutations have features that fall between those of the UPD and deletion groups. They exhibit microcephaly and seizures but are not hypopigmented. Their speech and motor skills are generally better than those seen in the deletion group [67]. Patients with AS satisfy the criteria for the diagnosis of autism.

On the contrary, duplications at 15q11–q13 (usually seen in females) are associated with up to 2% of cases of ASD with additional features of emotional lability, anxiety, hyperactivity dysmorphic facies, and seizures [68].

Epigenetic interactions between ASD and NDD conditions

Because RTT and AS show phenotypic and genetic overlap with autism, it was hypothesized that there is interaction along a common pathway. MeCP2 encodes methyl-CpG-binding protein 2, which acts as a transcriptional repressor for methylated gene constructs but is surprisingly not required for maintaining imprinted gene expression. Samaco et al. [46] tested the hypothesis that MeCP2 deficiency may affect the level of expression of UBE3A and neighboring autism candidate gene GABRB3 without necessarily affecting imprinted expression using an automated quantitation of immunofluorescence and *in situ* hybridization by laser scanning cytometry on tissue microarrays. They demonstrated significant defects in UBE3A/E6AP expression in two different Mecp2-deficient mouse strains and human RTT, AS, and autism brains compared with normal unaffected controls. Although no difference was observed in the allelic expression of several imprinted transcripts in Mecp2-null brain, they found that the UBE3A sense expression was significantly reduced, consistent with a reduction in the protein. They also found that a non-imprinted gene from 15q11–q13, *GABRB3*, encoding the beta 3 subunit of the $GABA_A$ receptor, demonstrated significantly reduced expression in multiple RTT, AS, and autism brain samples and Mecp2-deficient mice by quantitative immunoblot. Their results suggest an overlapping pathway of gene dysregulation within 15q11–q13 in RTT, AS, and autism and implicate MeCP2 in the regulation of UBE3A and GABRB3 expressions in the postnatal mammalian brain [46]. (See Figure 19.5.)

X-Linked disorders associated with ID

The expression of genes for X-linked disorders can be modulated by epigenetic control mechanisms leading to ID. Non-random X inactivation can result in tissues in which one cell type dominates in females. Here the X chromosome derived from the mother is the active one in most cells rather than there being an equal number of cells containing maternally derived and paternally derived active X chromosomes. Skewed inactivation has been documented as the cause of NDDs in heterozygous females with a number of X-linked conditions, including the urea cycle disorder ornithine transcarbamylase deficiency and certain forms of muscular dystrophy. It is also possible that a functional disomy of X-linked genes can occur in females who have structurally abnormal X chromosomes that do not contain the X inactivation center [46]. Dose-dependent overexpression of X-linked genes that "escape" X inactivation can also account for ID in individuals with X-chromosome aneuploidic disorders (i.e., when there are extra or deleted copies of X chromosomes), such as Turner syndrome (X0) and Klinefelter syndrome (XXY).

Fragile X syndrome

Fragile X syndrome (FXS) is the most common inherited cause of ID. Boys and girls with FXS have a phenotype that includes a characteristic physical appearance, cognitive skill deficits, and impaired adaptive behaviors [50]. Many affected children satisfy the criteria for the diagnosis of autism. The prevalence of FXS for males with the full mutation is about 1:3600. Prevalence of the full mutation in females is estimated to be at least 1:4000 to 1:6000. FXS arises from an expansion of the number of cytosine–guanine–guanine (CGG) triplet repeats occurring within the initial 5′-translated region of the *FMR1* gene. Inheritance of the instability in CGG regions leads to expansion from the normal number of repeats (6–40) to a premutation state (50–200) or from a premutation state to full mutation (>200 repeats). The stability of the CGG repeat depends on the length of the repeat as well as the sex

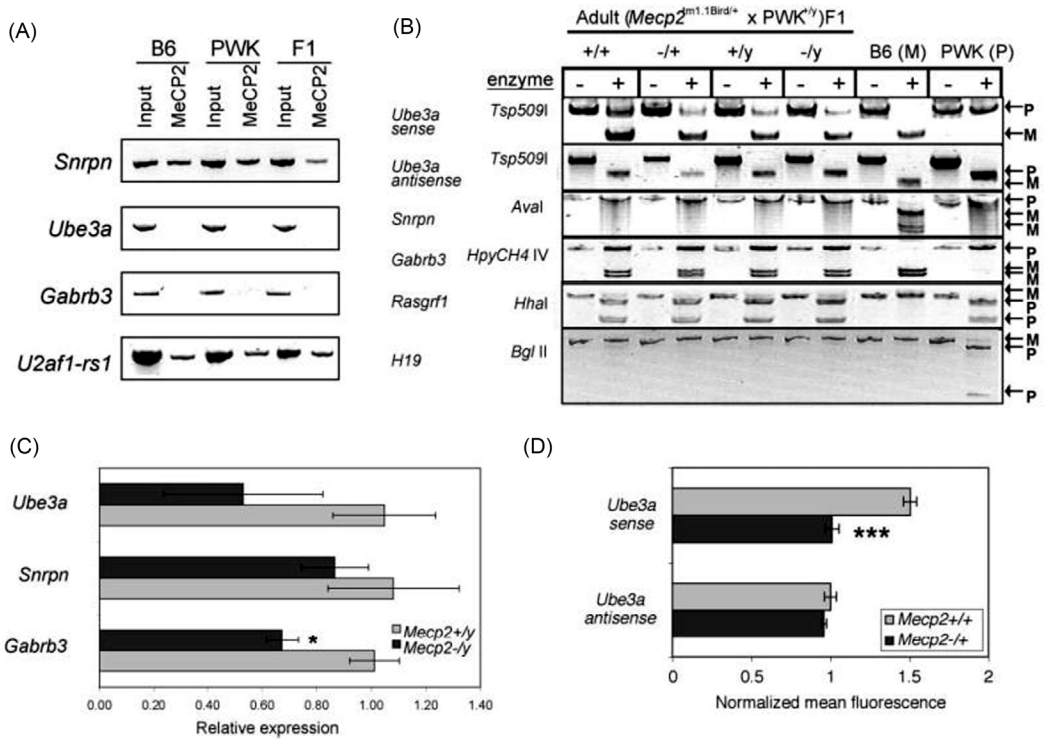

FIGURE 19.5

Imprinting and transcriptional analyses in MeCP2 mouse brain. (A) Chromatin from adult mouse cerebrum samples (C57B6, PWK, or (B6 × PWK)F1) was isolated for ChIP. Anti-MeCP2 (C-terminal) was used to immunoprecipitate DNA fragments from "Input" control. Ube3a and Gabrb3 promoters were not detected in the anti-MeCP2 precipitated chromatin, in contrast to the Snrpn promoter sequences that showed association with MeCP2. U2af1-rs1 was a positive control for a promoter previously demonstrated to bind MeCP2 in brain [34]. (B) Mecp2tm1.1Bird/+ (B6) females were crossed with wtPWK males to obtain F1 mice heterozygous for single nucleotide polymorphisms in the coding regions of several imprinted genes. RT-PCR followed by restriction enzyme digestion (+) was performed on RNA from adult brain samples of all Mecp2 genotypes as well as parental B6 and PWK samples (P, paternal; M, maternal). As reported previously for wt(B6a × PWK) F1 brain [67], Ube3a sense exhibited preferential maternal expression, Ube3a antisense and Snrpn were exclusively paternal, and Gabrb3 was biallelic, with no significant effect of Mecp2 genotype on these imprints. In addition, Rasgrf1 showed preferential paternal expression [68], whereas H19 showed exclusive maternal expression [69] in all samples. Results are representative of adult and neonatal F1 brain samples. (C) TaqMan PCR was used to quantitatively determine expression levels of Ube3a, Snrpn, and Gabrb3 in 10-week-old Mecp2–/y and Mecp2+/y brain RNA. Primers spanned intron/exon boundaries and are specific to the Ube3a sense transcript. Results shown are for the average ± SEM of four experimental replicates of two mice per genotype. Although Ube3a and Gabrb3 showed consistently lower expression in Mecp2-deficient brain compared with controls, Snrpn was not significantly changed. (D) Sense and antisense transcripts of Ube3a were detected by fluorescence *in situ* hybridization using single-stranded riboprobes and quantitated by LSC as described previously [16]. Results (mean ± SEM of 3 wt and 10 Mecp2–/ + samples) were normalized to control β-actin probe. $^{*}P < 0.05$, $^{***}P < 0.001$ by *t*-test.

of the individual passing on the mutation. The increased risk of CGG expansion from one generation to another is a phenomenon termed anticipation. The full mutation state results in hypermethylation of the promoter region of the *FMR1* gene. Methylation of a CpG island in a promoter region of a gene usually prevents expression of the gene (i.e., prevents transcription and translation). The number of trinucleotide repeats as well as the methylation changes in *FMR1* can be detected by clinically available molecular genetic blood testing. There are rare cases of males with an unmethylated full mutation (i.e., expanded repeat but lacking CpG methylation in the *FMR1* gene) who do not have ID [69].

These disorders profiled above demonstrate that epigenetic mechanisms are a significant modulator in varying syndromes that have in common neurocognitive disturbances. It has been hypothesized that due to the complexity of the human brain, precise spatial and temporal action of many genes is required and that a minimal alteration in this pattern is sufficient to cause a disturbance of integrated gene expression. In addition, the complex anatomy and cellular signaling mechanisms of the nervous system are noted to be associated with many more genes expressed as compared with other tissues. As this field continues to advance, understanding of how epigenetic pathways can regulate neuronal differentiation and maturation may reveal novel treatments (see below) [29,70–73].

Copy number variations

There has been an increased recognition that CNVs account for a number of NDDs [74]. This discovery has been enabled by advances in molecular cytogenetics, specifically microarray-based technologies. CNVs involve either: (1) DNA segments of 1 kilobase (Kb) or larger that are present in variable copy numbers in comparison to a reference genome, or (2) variations in the length of simple DNA triplet repeats, often referred to as "length-dependent variations of triplet repeats," which may involve only 150–180 bases (see also discussion below). CNVs tend to be more dynamic than point mutations, and they often occur *de novo*. They cause NDDs due to abnormal gene dosage. One particular type of CNV is the trinucleotide repeat expansion, which has been linked to a number of disorders that do not follow typical Mendelian inheritance. The first disorder discovered was FXS; others include Huntington's disease, myotonic dystrophy, Friedrich ataxia, dentatorubropallidal atrophy, and the spinocerebellar ataxias. Trinucleotide repeat disorders result from problems in recombination and replication during meiosis. The expansion length is linked to the phenotype, with the longer expansions presenting with the more severe clinical signs and symptoms. This is because the trinucleotide repeat expansion interferes with gene expression [75].

Microdeletion syndromes

Microdeletion syndromes involve chromosomal deletions that span several genes but are too small to be detected using conventional cytogenetic techniques. Homologous recombination of flanking low-copy repeat gene clusters is the cause of many of these disorders [52]. Such low-copy repeats, now called duplicons, flank genomic regions that are prone to deletion, duplication, and inversion. The features of microdeletion syndromes such as Smith–Magenis syndrome (SMS), Williams syndrome (WS), and DeGeorge/velocardiofacial syndrome (VCFS) are due to these gene dosage effects. This involves the same mechanism as in imprinted disorders such as AS and PWS [62,65].

Smith–Magenis syndrome

Smith–Magenis syndrome (SMS) is a disorder manifest by multiple congenital anomalies and ID [76]. The clinical phenotype includes rather distinctive craniofacial and skeletal features that change

with age, a history of infantile hypotonia, significant expressive language delay, ID, stereotypies, behavioral problems, and a sleep disorder because of an abnormal circadian secretion pattern of melatonin [54,55]. Many affected children satisfy the criteria for the diagnosis of autism. Recently, it has been determined that two genetic mechanisms contribute to SMS: an interstitial deletion involving chromosome 17p11.2 (including the retinoic acid-induced 1 gene) and a mutation in the retinoic acid-induced 1 gene.

Velocardiofacial syndrome

Velocardiofacial syndrome (VCFS) is an autosomal dominant condition caused by a 3-Mb deletion of contiguous genes on chromosome 22q11.2 [77]. Multiple organ systems are affected, including the face, palate, and heart. The condition has been referred to by various names including VCFS, Shprintzen syndrome, DiGeorge syndrome, conotruncal face anomalies syndrome, and CATCH 22 deletion. The VCFS syndrome presents with a highly variable clinical expression pattern that encompasses several different clinical presentations. The prevalence of VCFS is estimated to be about 1:4000, although this might represent an underestimation because of lack of case ascertainment. The 22q11.2 deletion is diagnosed in individuals who have a submicroscopic deletion of chromosome 22 that can be detected by FISH with DNA probes from the DiGeorge chromosomal region. Fewer than 5% of patients with clinical symptoms of the 22q11.2 deletion syndrome have normal routine cytogenetic analysis and negative FISH testing. At least 30 genes are encoded in the involved region of DNA. Research focusing on the neurobehavioral and cognitive aspects of VCFS delineate a nonverbal learning disability. Cognitive problems are present in the majority of individuals [78,79], and many affected children satisfy the criteria for the diagnosis of autism.

Williams syndrome

Williams syndrome (WS) is a chromosomal microdeletion syndrome characterized by a specific phenotype consisting of cognitive impairment in association with a characteristic cognitive profile, unique personality characteristics, distinctive facial features, and cardiovascular disease. In addition, a range of connective tissue abnormalities and multiorgan anomalies is observed. Hypercalcemia and hypercalciuria may be present in infancy [80]. WS can be transmitted as an autosomal dominant disorder. Both females and males are affected equally. Most cases occur *de novo*, but parent-to-child transmission has been observed, and there is a 50% risk of transmitting the deletion to offspring. Clinical diagnostic criteria are available for WS; however, the most reliable means for diagnosis relies on detection of the contiguous gene deletion of the WS critical region on chromosome 7q11.2 that encompasses the elastin (ELN) gene as well as numerous genes believed to contribute to the unique phenotype. Virtually all individuals with the clinical diagnosis of WS have been found to have this contiguous gene deletion, which can be detected using FISH [81]. The origin of the deletion can be paternal or maternal, without any parent-of-origin effect on the phenotype. Many of the clinical manifestations of WS are due to hemizygosity (one copy) of the elastin gene. Lim kinase 1 (LIMK1), a gene contiguous to ELN, is a second gene implicated in the WS phenotype [82,83].

Phelan–McDermid syndrome

Several other important examples of an increased risk for NDDs due to over- or underexpression of the same gene (or sets of genes) include 22q13, Phelan–McDermid syndrome [84]. Phelan–McDermid syndrome is a more recently recognized disorder featuring ID, significant language impairment, poor social communication, hypotonia, and dysmorphic facies [21]. It is due to a

microdeletion on chromosome 22q13 which leads to haploinsufficiency of the *SHANK3* gene [84]. The corresponding protein, SHANK3, stabilizes a protein complex including Homer, actin, and post-synaptic density protein 95. This complex plays an important role in dendritic spine formation and synaptic plasticity. In addition, it is required for the coordination of neurexin–neuroligin trans-synaptic signaling and the control of glutamatergic transmission via metabotropic glutamate receptor 5 (mGluR5), *N*-methyl-D-aspartate (NDMA), and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [85,86]. Studies in mice harboring the SHANK3 deficiency show autistic features and synaptic abnormalities [87–89]. In humans, deletions as well as truncating mutations of SHANK3 have been reported in ASD and schizophrenia, whereas microduplications have been associated with a milder phenotype consisting of ID, dysmorphic features, and ASD features [90,91]. SHANK3 is physically located upstream of the phosphatidylinositol 3-kinase (PI3K)/atypical kinase (AKT) pathway which plays a role in mTOR signaling.

The previous disorders illustrate how knowledge of ASD can point to common pathways. For example, the mTOR pathway plays central roles in synaptic protein synthesis [92,93], but because many of the ASDs are due to synaptic deficits, it is interesting that new data have revealed a role for the downstream mTOR pathway in autism [94]. mTOR is a key signaling node coordinating cell-cycle progression and cell growth in response to genetic and epigenetic factors, and perturbed mTOR-dependent mRNA translation may be involved in FXS due to overactivity of mTOR. mTOR may be a target for treatment in NDDs and ASDs because in the brain components of the mTOR pathway are present at synapses, where they regulate dendritic spine morphology and are essential for synaptogenesis [92,95]. (See Figure 19.6.)

Epigenetic hot spots in autism

The Autism Genome Project Consortium performed single nucleotide polymorphism (SNP) and CNV analyses of 1181 families, the largest analysis of its kind for ASD, demonstrating a suggestive linkage peak on chromosome 11p12–p13 for all families and CNVs within autism susceptibility candidate loci.

Chromosomes 15q and 7q

Due to the wide use of genome-wide scans of affected sib-pairs with autism or ASD, several loci have been identified as being hot spots for ASD. In particular, chromosome loci 1p, 2q, 3p, 7q, 15q and 17q show the strongest evidence based on replication in independent studies of large autism patient cohorts [96,97]. Several of the linkage peaks overlap or are in close proximity to regions that are subject to genomic imprinting. The most widely studied are on chromosomes 15q11–q13, 7q21–q31.31, and 7q32.3–q36.3, demonstrating evidence for a combination of genetic as well as epigenetic factors that confer risk for ASD. (See Figure 19.7.)

Chromosome 15

Human chromosome 15q11–q13 is a complex locus that harbors imprinted and non-imprinted genes. The imprinted genes are subject to strict copy number and parent-of-origin rules as previously discussed in the section on PWS and AS. Deletion or duplication of 15q11–q13 $GABA_A$ receptor subunit (GABR) genes occurs in multiple human neurodevelopmental disorders including PWS, AS, and autism. In addition to the imprinted genes is a cluster of three GABR genes: *GABRB3*, *GABRA5*, and *GABRG5*. GABRB3 protein expression is also reduced in RTT, caused by mutations in MeCP2 on Xq28. In a series of experiments, Hogart et al. [98] showed that all three GABR genes are

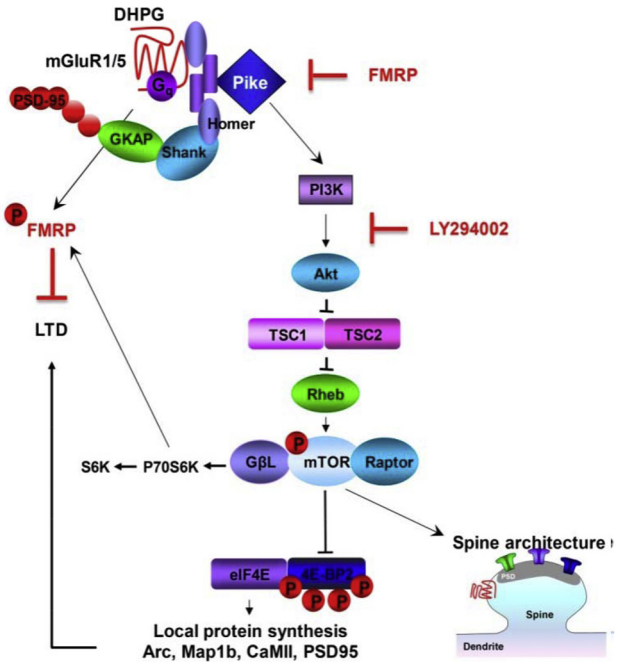

FIGURE 19.6

The link between deficits in fragile X mental retardation protein (FMRP) and overactivated mTOR. The findings support a model where, in WT mice, FMRP represses PIKE, an upstream activator of PI3K signaling and identified target of FMRP, and thereby inhibits mTOR signaling.

biallelically expressed in 21 control brain samples. In order to conclusively determine if 15q11–q13 $GABA_A$ receptor genes exhibit imprinted expression, they analyzed allelic expression in control brain samples from the frontal cortex, Brodmann area. Similarly, they included RTT and autism brain samples to determine whether there was epigenetic dysregulation and they assessed the copy number of chromosome 15q11–q13 using DNA FISH with the GABRB3 probe which they hybridized to brain samples on a tissue microarray. They found normal disomic 15q11–q13 expression in control, RTT, and autism brain samples, suggesting that these genes are not imprinted in the normal human brain. However, in eight autism and one of five RTT brain samples there was monoallelic or highly skewed allelic expression of one or more GABR genes, suggesting that epigenetic dysregulation of these genes is common to both disorders. Quantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis of PWS and AS samples with paternal and maternal 15q11–q13 deletions revealed a paternal expression bias of GABRB3, while RTT brain samples showed a significant reduction in GABRB3 and UBE3A. These results suggest that MeCP2 is acting to regulate expression of both alleles of GABRB3 in neurons [99].

Duplication of 15q11–q13 Maternal interstitial duplications of chromosome 15q11–q13 account for a small percentage (1–2%) of ASD cases [99]. These rearrangements may be due to an interstitial

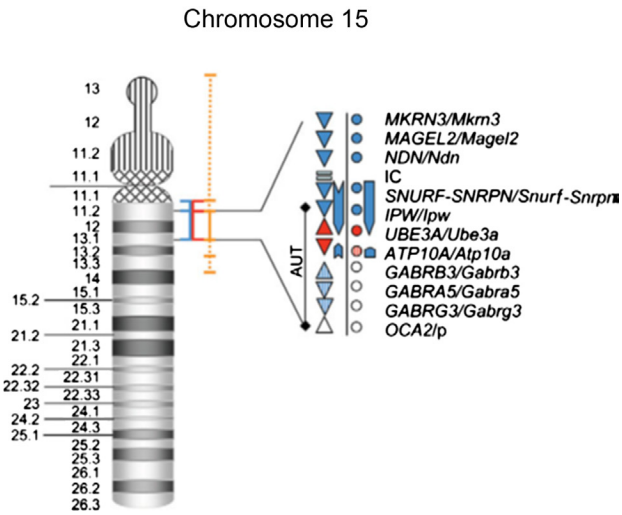

FIGURE 19.7

Ideogram of chromosome 15.

duplication, int dup(15), or a supernumerary pseudodicentric chromosome 15, idic(15). This locus involves the imprinted genomic region that is deleted in PWS and AS. The behavioral phenotype of patient harboring maternal 15q11–q13 duplications demonstrated a spectrum of cognitive impairments, dysmorphic facial features, seizures, and a number of neurobehavioral traits such as anxiety, emotional lability, tantrums, and hyperactivity [100]. A maternal supernumery derivative chromosome 15 containing two copies of the 15q11–q13 region and two centromeres (isodicentric chromosomes 15) is also associated with increased ASD risk. The increased risk of ASD with 15q11–q13 maternal duplication and with the PWS molecular subtype with maternal uniparental disomy suggests an important role of the maternally expressed gene UBE3A. Overexpression of UBE3A was also observed in lymphoblastoid cell lines from patients with 15q11–q13 duplication, whereas the paternally expressed genes MKRN3, MAGEL2, and SNRPN were expressed at levels similar to those measured in control individuals [101,102]. However, expression profiling in the brain tissue of two ASD patients with maternal 15q11–q13 duplication indicated that the mechanism underlying ASD susceptibility is more complex than just UBE3A upregulation [103]. Hogart et al. [103] evaluated postmortem brain tissue in a male found to have a rare 15q11–q13 hexasomy and a female with 15q11–q13 tetrasomy. They used quantitative RT–PCR to measure 10 15q11–q13 transcripts in maternal 15q11–q13 duplication, PWS, and control brain samples. They found that gene expression and methylation status correlated with parental gene dosage in the male, but in the female with autism and milder PWS-like characteristics there were deficiencies rather than increased dosage of paternally expressed transcripts SNRPN, NDN, HBII85, and HBII52 and unchanged levels of maternally expressed UBE3A compared with controls, suggesting that chromosome 15q11–q13 duplication syndrome brain reveals epigenetic alterations in gene expression that were not predicted from copy number.

Imprinting and epigenetics of chromosome 7q in ASD

One of the first regions identified in linkage studies in ASD encompassed most of the long arm of chromosome 7q. Additionally, single families have been identified among whom an AD family has been identified in which three siblings inherited from their mother a paracentric inversion in the chromosome 7 candidate region, inv(7) (q22–q31.2) [104]. Because communication issues are a core feature of ASD, a number of research groups performed linkage studies to identify genes that contribute to both autism and SLI. Loci on 7q31 may be important in the genetic etiologies of autism and SLI as has been suggested in four independent linkage studies [7,104]. Several genes have been identified, including the autism susceptibility locus on human chromosome 7q32 which contains the maternally imprinted mesoderm-specific transcript (MEST) homolog protein, which encodes a member of the [alpha]/[beta] hydrolase fold family and has isoform-specific imprinting, and the non-imprinted coatomer protein complex, subunit gamma 2 (COPG2), which interacts with dopamine receptor D1, as well as TSGA14, which encodes a centrosomal protein gene [105–108]. DNA methylation at these promoter regions in the autism susceptibility locus on human chromosome 7q32 has been highly conserved during primate evolution, but COPG2 shows human-specific downregulation in the brain.

Chromosome 16

Recurrent 16p11.2 microdeletion was initially described in individuals with ASD [109–111] but is now recognized to comprise a spectrum which is characterized by developmental delay, ID, and/or ASD. IQ scores may be in the normal to mild intellectually impaired range, but expressive language is affected [112]. Individuals with 16p11.2 microdeletion are at increased risk for seizures and/or EEG abnormalities. Most cases have come to light due to increased use of chromosome microarrays. Prior to that, the same 16p11.2 microdeletion was reported as a *de novo* copy number variant in an individual with Asperger disorder [113] and in two monozygotic twins [114]. Weiss et al. [115] proposed that this deletion does not involve 100% penetrance for a strictly defined form of autism but instead represents a highly penetrant cause of developmental disability. In addition to deletion of 16p11.2, duplication of one copy of the human 16p11.2 interval is also associated with encephalopathy, including ASD, ID, and other phenotypes. Thus, this region needs to have intact gene dosage. The core of this CNV includes 25 genes. Most genes in this region are required for nervous system development [111].

Can drugs be developed for NDDs based on epigenetics?

Understanding epigenetic mechanisms and the disorders reviewed herein can provide important insights into designing therapies for ASD and NDDs. Key regulatory regions may serve as important targets for drug action. For example, given the role that DNA methylation plays in several NDDs, DNA-demethylating drugs should be investigated as having a potential role in several neurodegenerative and NDDs, including FXS and Rubinstein–Taybi syndrome. Methylation of DNA acts to repress gene expression in imprinted domains and also provides a mechanism through which environmental factors can have long-lasting effects on the genome. In order for drugs affecting DNA methylation to be effective over the life of an individual with an NDD, dynamic reversibility is essential. Preclinical cell culture and animal model data support the dynamic, reversible DNA methylation pattern in the brain and on memory [116]. Furthermore, Tabolacci et al. [117] showed that treatment

with acetyl-L-carnitine (ALC), a compound that reduces the *in vitro* expression of the FXS fragile site without affecting DNA methylation status, caused an increase of H3 and H4 acetylation but not H3–K4 methylation, suggesting that H3–K4 methylation and DNA demethylation are the main epigenetic controls activating *FMR1* gene expression. Histone acetylation appeared to play an adjunctive role. DNA methylation inhibitors are able to reactivate the expression of previously silenced genes. Pyrimidine nucleoside analogs of cytidine (e.g., 5-azacytidine, 5-aza-2-deoxycytidine) are hypomethylating agents that inhibit DNA methylation by incorporation into cytosine residues in replicating DNA. This leads to trapping of DNA methyltransferases and subsequently to demethylated DNA. They are, however, cytotoxic, which is advantageous in cancers but not developmental disorders [118,119]. Chiurazzi et al. [120] showed that treatment of lymphoblastoid cells of patients with FXS with 5-aza-2-deoxycytidine led to reactivation of fully mutated *FMR1* genes with 300–800 repeats, as shown by the restoration of specific mRNA and protein production. This effect also correlated with the extent of promoter demethylation.

Most epigenetic treatments in NDD have focused on histone acetylation because there are several elements that make it an attractive target: (1) it is dynamically modulated and better than DNA methylation for controlling gene transcription; (2) there is strong evidence for a key role of histone acetylation in the control of cognitive processing, which is a major aspect of the NDDs [121,122]; and (3) a genetic loss of HAT is causal to at least one of the conditions covered in this review (Rubinstein–Taybi syndrome), and alterations in histone acetylation. Valproate, an HDAC inhibitor and a drug used for the treatment of epilepsy and bipolar disorder, affects a number of signaling pathways and was originally touted as an inhibitor of GABA transaminase due to its ability to enhance GABA availability [123]. Valproate is an inhibitor of HDAC classes I and II and causes H3 and H4 acetylation in astrocytes. In animal models, the administration of valproate was followed by increased levels of acetylated H3 and H4 in the brain. Toriolli et al. [124] studied the effect of valproic acid (VPA) on inattention in FXS. They chose to study this agent as it is described as an inhibitor of HDAC and, possibly, as a DNA demethylating agent. They studied the *in vitro* response of lymphoblastoid cells from FXS patients treated with VPA. The authors found that the administration of VPA to the cells caused a modest reactivation of *FMR1* transcription and increased levels of histone acetylation, confirming the histone hyperacetylating effect, but not its putative DNA demethylating activity. The *in vivo* efficacy of VPA was assessed on symptoms of ADHD in FXS patients [125]. Overall, they observed an improvement in adaptive behavior, defined as the performance of daily activities required for personal and social competence, due to a significant reduction in hyperactivity after VPA treatment. More studies will be needed to determine long-term efficacy. The HDAC inhibitors sodium butyrate, phenylbutyrate, trichostatin A, and suberoylanilide hydroxamic acid are being used in preclinical studies of Rubinstein–Taybi syndrome. Mouse models that have been treated with HDAC inhibitors show improvement in long-term memory deficits [126,127].

Increased knowledge about the mechanisms of gene silencing and epigenetic regulation in FXS has generated interest in new drugs for this disorder. Studies in mice have revealed that core defects could be corrected by downregulation of metabotropic glutamate receptor 5 (mGluR5) signaling [128]. This was based on the theory that absence of the protein due to gene silencing by methylation leads to exaggerated protein synthesis after mGluR5 activation and receptor internalization. A human study using the selective mGluR5 antagonist fenobam led to decreased hyperactivity and anxiety in adults with FXS [129].

Conclusions

Several epigenetic models of autism have been proposed. These are suggested by the occurrence of autism in individuals with FXS, which arises from epigenetic mutations, and with RTT, which involves epigenetic regulatory factors. A number of chromosome loci with and without identified genes or susceptibility loci have been identified. Understanding the underlying epigenetic mechanisms allows consideration of treatment targets for developmental disorders. The consequences of these NDDs suggest that epigenetic mechanisms and chromatin remodeling need to be tightly controlled for proper cognitive function to occur. Dysregulation can have devastating consequences on neurodevelopment. Because epigenetic mechanisms are dynamic, they are also potentially reversible and may be manipulated by pharmacological interventions [130,131].

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Glossary

Alpha thalassemia/mental retardation syndrome X-linked (ATRX) A chromatin-remodeling factor and DNA methylation modifier that leads to an X-linked disorder if there is a mutation. Mutations in this gene cause the alpha thalassemia/mental retardation syndrome, which is manifest by intellectual delays, facial abnormalities, urogenital defects, and alpha-thalassemia.

Aneuploidic disorders Characterized by extra or deleted copies of X chromosomes.

Angelman syndrome Complex genetic disorder that affects the nervous system. Characteristic features of this condition include delayed development, intellectual disability, severe speech impairment, and problems with movement and balance (ataxia). Most affected children also have recurrent seizures (epilepsy), a happy disposition, and microcephaly. Delayed development becomes noticeable by the age of 6 to 12 months, and other common signs and symptoms usually appear in early childhood.

Autism spectrum disorders A range of conditions classified as neurodevelopmental disorders in *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition.

Brain plasticity and synaptic connectivity Changes in dendritic spine morphology and branching.

Cyclin-dependent kinase-like 5 (CDKL5) Gene that is a member of the Ser/Thr protein kinase family and encodes a phosphorylated protein with protein kinase activity. Mutations in this gene have been associated with X-linked infantile spasm syndrome (ISSX), also known as X-linked West syndrome and Rett syndrome (RTT).

Coffin–Lowry syndrome A neurological disease caused by a deficiency in a histone phosphorylase. An X-linked disorder, this syndrome is associated with severe ID and a dysmorphic facial appearance. The most salient features include a prominent forehead, down-slanting palpebral fissures, orbital hypertelorism, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Coffin–Lowry syndrome is caused by loss-of-function mutations in the RSK2 gene.

Fragile X syndrome (FXS) Most common inherited cause of ID. Boys and girls with FXS have a phenotype that includes a characteristic physical appearance, cognitive skill deficits, and impaired adaptive behaviors.

Many affected children satisfy the criteria for the diagnosis of autism. The prevalence of FXS for males with the full mutation is about 1:3600. Prevalence of the full mutation in females is estimated to be at least 1:4,000–1:6000. FXS arises from an expansion of the number of cytosine–guanine–guanine (CGG) triplet repeats occurring within the initial 5′ translated region of the *FMR1* gene (fragile X mental retardation protein gene).

Neurodevelopmental disabilities Chronic conditions that affect developing and mature nervous system such as cerebral palsy, mental retardation, behavioral syndromes, or neurologic conditions.

Phelan–McDermid syndrome Phelan–McDermid syndrome is a more recently recognized disorder featuring ID, significant language impairment, poor social communication, hypotonia, and dysmorphic facies. It is due to a microdeletion on chromosome 22q13 which leads to haploinsufficiency of the *SHANK3* gene.

Prader–Willi syndrome Complex genetic condition that affects many parts of the body. In infancy, this condition is characterized by weak muscle tone (hypotonia), feeding difficulties, poor growth, and delayed development.

Rapamycin (mTOR) signaling A protein kinase that controls cell growth, proliferation, and survival.

Rasopathies Clinically defined group of medical genetic syndromes caused by germ line mutations in genes that encode components or regulators of the Ras/mitogen-activated protein kinase (MAPK) pathway. These disorders include neurofibromatosis type 1, Noonan syndrome, Noonan syndrome with multiple lentigines, capillary malformation–arteriovenous malformation syndrome, Costello syndrome, cardiofaciocutaneous syndrome, and Legius syndrome.

Rett syndrome/MeCP2 A neurodevelopmenal disorder that affects girls almost exclusively. It is characterized by normal early growth and development followed by a slowing of development, loss of purposeful use of the hands, distinctive hand movements, slowed brain and head growth, problems with walking, seizures, and intellectual disability.

Rubinstein–Taybi syndrome Syndrome associated with the dysfunction of a histone acetyltransferase. An autosomal dominant disorder, it is characterized by ID and physical anomalies, most notably broad radially deviated thumbs and phalluses and a postnatal growth deficiency that is later followed by excessive weight gain. There are also characteristic dysmorphic facial features and an increased risk for developing tumors. Rubinstein–Taybi syndrome is caused by mutations in the cAMP response element-binding (CREB) protein (CBP) gene.

Smith–Magenis syndrome Disorder manifest by multiple congenital anomalies and ID. The clinical phenotype includes rather distinctive craniofacial and skeletal features that change with age, a history of infantile hypotonia, significant expressive language delay, ID, stereotypies, behavioral problems, and a sleep disorder because of an abnormal circadian secretion pattern of melatonin synaptopathies.

***UBE3A* gene** Gene that encodes ubiquitin protein ligase E3A. This enzyme is involved in targeting proteins for degradation within cells. Both copies of the *UBE3A* gene are active in most of the body's tissues, but in the brain only the maternal copy is normally active. If this copy is mutated or lost, it affects many developmental processes including Angelman syndrome.

Velocardiofacial syndrome Autosomal dominant condition caused by a 3-Mb deletion of contiguous genes on chromosome 22q11.2. Multiple organ systems are affected, including the face, palate, and heart.

Williams syndrome Microdeletion syndrome characterized by a specific phenotype consisting of cognitive impairment in association with a characteristic cognitive profile, unique personality characteristics, distinctive facial features, and cardiovascular disease. In addition, a range of connective tissue abnormalities and multiorgan anomalies is observed. Hypercalcemia and hypercalciuria may be present in infancy.

CHAPTER

Epigenetics in Child Psychiatry

20

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KEY CONCEPTS

- The study of the role of epigenetics in child psychiatric disorders is in its early stages. Epigenetics may help unravel the complexities of these disorders.
- Epigenetics contributes to the mechanism of action of drugs used to treat child psychiatric disorders.

ABBREVIATIONS

ADHD Attention deficit hyperactivity disorder
apoD Apolipoprotein D
ATP Adenosine triphosphate
BDNF Brain-derived neurotrophic factor
bHLH Basic helix–loop–helix
Cdkn1c Cyclin-dependent kinase inhibitor 1c gene
CpG Cytosine–phosphate–guanine
CPN Central pretectal nucleus
DAT Dopamine transporter
DBP D-binding protein
DHA Docosahexaenoic acid
DRD4 Dopaxmine receptor 4 gene
DZ Dizygotic
EAAT2 Excitatory amino acid transporter 2
FMR1 Fragile X mental retardation gene 1
FXS Fragile X syndrome
GLT1 Glutamate transporter 1
GRM1 Glutamate receptor, metabotropic 1
H3K9 Histone H3 lysine 9
HDAC Histone deacetylase
hERG Human ether-a-go-go related gene
IEGs Immediate early genes
LSD1 Lysine-specific demethylase 1
MAO Monoamine oxidase
mCpG Methyl-CpG
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
mRNA Messenger RNA
MZ Monozygotic
NAc Nucleus accumbens
NET Norepinephrine transporter
NF-κB Nuclear factor κ-light-chain-enhancer of activated B cells
NGFIB Nerve growth factor-inducible B
NMDA *N*-methyl-D-aspartate
PFC Prefrontal cortex
SNARE Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
tRNA Transfer RNA
VGLUT1 Vesicular glutamate transporter 1
VNTR Variable number of tandem repeats

Introduction

Epigenetics, as defined by the National Institutes of Health (NIH) Roadmap Epigenomic Project, refers to changes in the regulation of gene activity and expression that are not dependent on gene sequence. Research in this rapidly expanding field is proving critical in psychiatric disorders that do not follow a simple linear gene–phenotype. The search for genes conferring risk for psychiatric disorders such as attention deficit hyperactivity disorder (ADHD) and schizophrenia has thus far identified some common variants with small effects [1,2] and rare variants with large effects [3,4]. However, these risk variants cannot account for the total risk, the differing phenotypic presentations within the same family or within the same individual during different developmental stages, or the discordance in monozygotic (MZ) twins. Epigenetic research is beginning to elucidate some of the synergistic effect between genes and factors impacting on genes. In this chapter, we will review the literature on epigenetic mechanisms, and explore their potential roles in the pathophysiology of the child psychiatric disorders ADHD and autism, as well as the psychotropic drugs used to treat child psychiatric disorders.

Epigenetic mechanisms

The major processes whereby genes are expressed or silenced in psychiatric disorders include DNA methylation [5], histone modifications [6], transcription factors [7], and posttranscriptional regulation of gene expression through miRNAs [8]. Most epigenetic changes are limited to an individual over the course of his/her lifetime; however, epigenetic changes that cause DNA mutations in progeny cells are inherited from one generation to the next [9].

DNA methylation

The human DNA library is composed of over 3 billion base pairs, with adenine–thymine (ApT) and cytosine–guanine (CpG) being bound by a phosphate. Cytosine is methylated (mCpG) in about 2–6% of the CpG dinucleotide pairs, which usually, but not always, results in transcriptional repression. Wu and colleagues [10] have shown that, although methylation of DNA sequences in promoters tends to be repressive, methylation of DNA sequences beyond the promoters can actually promote gene expression. In the human brain, methylation also occurs in non-CpG nucleotides where G is replaced by A, C, or T and these forms are also thought to inhibit transcription [11]. Lister and colleagues [12] investigated DNA methylation across the entire genomes in human frontal cortex throughout the lifespan and found non-CpG methylation as the predominant form of methylation accounting for 53% versus 47% CpG in neurons. As can be seen in Table 20.1, methylation of the neuronal genome was found to increase during the first two years after birth followed by a slower accumulation during adolescence paralleling synpatogenesis and continuing during synaptic pruning. Interestingly, the level of methylation in glia was minimal, comparable with that noted in fetal and postnatal cortical tissue. Some genes escaping X-inactivation were also discovered.

Chromosome organization

Chromatin consists of DNA, histone proteins, and non-histone proteins. DNA is coiled around histone proteins that are organized in sets of eight (nucleosome), and when chromatin is condensed it is inactivated (heterochromatin), preventing transcription. It can open to an active state (euchromatin) by various mechanisms, including methylation, acetylation, phosphorylation, ubiquitylation, and SUMOylation [6,15]. Some chromatin where histones and DNA are methylated and bound to repressor proteins are never accessible to transcription. Other portions of chromatin are in "repressed" or "permissive" states to facilitate critical processes such as cell division or to modulate the regulation of neural developmental maturation.

Transcription factors

For a gene to carry out a function it needs to be expressed, meaning that the DNA needs to be transcribed to RNA [7]. This process occurs in the nucleus of eukaryotic cells and is mediated by positive and negative regulatory proteins (transcription factors) that bind to specific regions of the DNA and stimulate or inhibit transcription [16]. Basal transcription factors are proteins that facilitate the proper alignment of RNA polymerase on the DNA template strand, while special transcription factors such as those involved in the regulation of heat-, light-, and hormone-inducible genes bind to enhancers located in the vicinity of a gene [17].

Table 20.1 Human Brain Maturation and Epigenetic Reconfiguration

Study	Prenatal (weeks)									Birth	Postnatal (months)					Postnatal (years)							Adult
	1	4	8	12	16	20	24	28	32		2	3	4	5	6	1	2	5	6	12	16	18	
Giedd et al. [13]	**Neurulation**																						
		Neuron proliferation																					
				Neuron migration																			
					Apoptosis																		
								Myelination															
Huttenlocher and Dabholkar [14]						**Synaptogenesis**																	
														Synaptic pruning									
Lister et al. [12]										**Cortical neuronal methylation 50%**					**Cortical neuronal methylation 75%**								

Posttranslational regulation

As reviewed by Tardito and colleagues [18], unlike messenger RNA (mRNA), which is transcribed from DNA to interact with transfer RNA (tRNA) to form protein, microRNA (miRNA), which was discovered in 1993, refers to a group of small (20–22 nucleotides) RNA molecules that do not encode information but are present in numerous organisms of varying cellular complexity. They are typically at least partially complementary to mRNA sequences and as such act as posttranscriptional modifiers of protein synthesis by inactivation or activation of specific sequences. The miRNAs inhibit protein production by suppressing the translation process or via deadenylation (e.g., removing the poly A tail), thereby effecting mRNA degradation. In some cases however, miRNAs may activate translation of protein products. In mammals, miRNAs may control the activity of up to 50% of protein encoding genes involved in nearly every cellular function and may be expressed in a tissue-specific or developmental stage-specific manner. It is therefore theorized that alterations in the function or expression of miRNAs could have major implications for the pathogenesis of many human diseases, including child psychiatric disorders; furthermore, by acting at the level of the miRNA, some pharmacological interventions could have major implications for improving the treatment of these conditions [18].

Epigenetic processes and child psychiatric disorders

The epigenetic processes involved in most child psychiatric disorders remain to be elucidated. Significant progress has been made in two disorders: Rett syndrome and fragile X syndrome (FXS). In Rett syndrome, the loss of developmental milestones has been attributed to abnormal gene expression in the brain caused by a lack of a normal MeCP2 protein that recognizes methylated DNA and helps to repress gene expression [19]. FXS, the most common cause of mental retardation, is attributed to hypermethylation of DNA at the FMR1 and FMR2 promoters, caused by trinucleotide repeat expansion [20]. Table 20.2 summarizes some of the epigenetic mechanisms hypothesized to play a role in two major child psychiatric disorders: ADHD and autism. More details on the epigenetic aspects of Rett syndrome and FXS are given in Chapter 19.

Attention deficit hyperactivity disorder

As reviewed by Archer and colleagues [26], many of the same environmental risk factors conferring risk for schizophrenia also have been reported to confer risk for ADHD. Variants of the dopamine transporter gene (*DAT; SLC6A3*) have been associated with ADHD. This gene also has features that render it vulnerable to epigenetic factors, including the large number of variable number of tandem repeats (VNTRs), which may indicate a tendency for chromatin structure to remain highly accessible to modifiers; the high GC density throughout the gene, especially in its promoter sequence; and the several transcription factors, including sp1 which mediates hormone-dependent gene activation [21]. Further suggestive modifications from epigenetic factors include the decreased *DAT* mRNA expression reported with age [27] and in response to drugs [28], environmental factors [28], and pathogens [29]. Wong and colleagues [23] measured DNA methylation across promoter regions of several other genes implicated in ADHD, such as the dopamine receptor 4 gene (*DRD4*), the serotonin transporter gene (*SLC6A/SERT*), and the monoamine oxidase (*MAOA*) gene in monozygotic and dizygotic twins at ages 5 and 10. DNA methylation differences were found early in childhood even between identical

Table 20.2 Epigenetic Mechanisms in ADHD and Autism

Mechanism	ADHD	Autism
DNA methylation	SLC6A3 is GC dense especially in its promoter [21] Methylation differences between MZ and DZ twins on several genes are implicated in ADHD (*DRD4*, *SLC6A/SERT*, *MAOA*) [23]	Hypermethylation of DNA at the FMR1 and FMR2 promoters, caused by trinucleotide repeat expansion in fragile X syndrome [20,22] Lack of normal MeCP2 protein that recognizes methylated DNA and helps to repress gene expression in Rett syndrome [19]
Transcriptional factors	Flanking sequences of *SLC6A3* contain binding sites for GATA-1, CREB, c-*Myc*, *cis*-acting regulatory elements [21]	—
Histone modification	Over 90 VNTRs in the *SLC6A3* gene body indicate a tendency to open chromatin structure and increase accessibility to modifiers [21]	—
microRNAs	Two sequence variants at the MiR-183-96-182 cluster contribute to ADHD [24]	miR-132 is known to be induced by endotoxins and regulate the immune response as well as to be affected by cytomegalovirus infection, which is known to contribute to autism pathogenesis [25]

Abbreviations: CREB, cAMP response element-binding protein; DZ, dizygotic; MZ, monozygotic; VNTR, variable number of tandem repeats.

twins, and the individual differences were not stable over time, implicating a significant role for environmental influences.

Low birth weight has been associated with ADHD in monozygotic birth-weight-discordant twin pairs [30–33], where the lighter twin from both the monozygotic (MZ) and dizygotic (DZ) birth-weight-discordant twins showed higher ADHD ratings [34]. The mechanism for this is not known; however, animal studies indicate overexpression of dopamine-related genes, including cyclin-dependent kinase inhibitor 1c (*Cdkn1c*), known to be critical for dopaminergic neuronal development, in the offspring of the mouse dams that had been given a diet deficient in proteins throughout pregnancy and lactation versus a control group that received adequate protein. Methylation of the promoter region of *Cdkn1c* was found to be decreased by half and Cdkn1c mRNA expression was increased across brain regions in these animals that were hyperactive and had altered reward processing [35]. In animal studies, subneurotoxic anoxia induced with nitrogen during postnatal days 1 to 7 in rats resulted in reduced expression of GRM1 and GRM5 mRNA levels in the hippocampus and cerebral cortex, resulting in inattentive symptoms and anxiety without any impairment in spatial learning or any detectable brain damage [36]. Two sequence variants at an miRNA cluster have also been reported in ADHD [24].

Autism

Epigenetic aspects of autism are briefly described in Table 20.2. The epigenetic aspects of autism and autism spectrum disorders are discussed in more detail in Chapter 19.

Psychotropic drugs and gene expression

The mechanisms by which psychotropic drugs reduce child psychiatric symptoms are not well understood. A growing body of research is showing that in addition to changing neurotransmitter activity, their activity may also be mediated through changes in gene expression in relevant neuronal pathways. Sprague–Dawley rats were treated with atomoxetine for consecutive postnatal days 21 to 41. Brains analyzed at day 42 were found to have decreased levels of the norepinephrine transporter (NET) in the hippocampus, and decreased levels of *N*-methyl-D-aspartate receptor (NMDAR) subunit 2B in both the striatum and hippocampus; however, transcript levels were unchanged. Surprisingly, 2 months after treatment was stopped, NMDAR protein levels were found reduced in the striatum and hippocampus and two SNARE proteins, synaptophysin and SNAP-25, were also altered [37]. D-amphetamine increases synaptic protein expression spinophilin in the striatum [38] and upregulates spinophilin and VGLUT1 in thalamus [39]. Neuroleptics may be improving psychotic symptoms by increasing glutamatergic action by downregulating glutamate transporter activity at both glial and neuronal sites. Clozapine downregulated EAAT2 (glial glutamate transporter) in rat hippocampus and parietal, temporal, and frontal cortex, and also downregulated EAAT3 (neuronal glutamate transporter) in striatum, hippocampus, and the cingulate, temporal, and frontal cortex. Haloperidol downregulated EAAT2 in cingulate and frontal cortex and reduced EAAT3 in nucleus accumbens, infralimbic cortex, and hippocampus [40]. Chronic clozapine exposure in mice led to increased mRNA encoding apoD in striatum, globus pallidus, and thalamus [41]. As reviewed by Le-Niculescu and colleagues [42], omega-3 fatty acids have been hypothesized to play a role in mood, aggression, alcohol use, and psychosis. Using DBP knockout mice subjected to a chronic stress paradigm they found that docosahexaenoic acid (DHA) modulates some of the same genes targeted by some of the current psychotropic medications and also increases myelin-related gene expression. For example, *GSK3B* expression was reported to be increased in postmortem human brains of depressed patients and inhibited by lithium; it was found to be decreased in mouse prefrontal cortex (PFC) by DHA.

Psychotropic drugs and epigenetics

Histone modifications

As shown in Table 20.3, haloperidol, clozapine, and risperidone have been shown to cause histone modifications. In rats, haloperidol decreased global DNA methylation levels [58]. Infusion of haloperidol into mice striatum caused phosphorylation of histone H3, acetylation of H3K14 [57], and phosphoacetylation that is considered to be mediated by the NMDA receptor [57]. Through acetylation of H3K14, haloperidol has been shown to induce a therapeutic effect [43]. Clozapine has been shown to increase promoter-associated H3K9 and H3K14 acetylation [48] as well as increase trimethlyation of H3K4, both of which lead to increased GABAergic activity [48] that may be partially responsible for its therapeutic effect. Huang and colleagues [51] reported that risperidone induced global phosphoacetylation of H3 in the striatum that was mediated by the NMDA receptor, suggesting that neurotransmitters such as dopamine, serotonin, and glutamate may act together to influence chromatin regulation. Antidepressants also influence histone modification. Imipramine has been shown to cause global H3 dimethylation [52]. In a study by Tsankova and colleagues [63], epigenetic regulation of the *BDNF*

Table 20.3 Epigenetic Mechanisms and Psychotropic Drugs

Histone modification	Antipsychotics	Antidepressants	Stimulants	Mood Stabilizers
Histone acetylation	Haloperidol: acetylation of H3 lysine [43] Clozapine: increase in promoter-associated H3K9 and H3K14 acetylation [48]	Imipramine: hyperacetylation of H3 at *BDNF* promoters [44] Amitriptyline: increased H3 acetylation by inhibiting HDAC activity [49] Fluoxetine: decreased acetylation of H3 in caudate putamen, frontal cortex, and hippocampus [50]	—	Valproic acid: increases levels of acetylated histone H3 and H4, promoting gene expression associated with hippocampal neurogenesis [45]; induces expression of reelin [46,47]
Histone methylation	Clozapine: increase in trimethlyation of H3K4, increase in GABAergic activity [51]	Imipramine: global H3 dimethylation [52] MAO inhibitors: demethylate dimethylated H3K4, thus inhibiting transcription [54,55] Fluoxetine: reverses decreased histone H3K9 trimethylation in hippocampus [56]	—	Valproic acid: demethylation of reelin promoter [48], global demethylation of CpG in astrocytes of the cortex and hippocampus [53]
Histone phosphorylation	Haloperidol: phosphorylation of histone H3 [57]	—	—	—
Histone phosphoacetylation	Haloperidol: phosphoacetylation mediated by NMDA receptor [43] Risperidone: global phosphoacetylation of H3 in the striatum mediated by NMDA receptor [43]	—	—	—

DNA methylation	Haloperidol: decreased global DNA methylation levels [58] Clozapine: reduced cytosine methylation at Gadd45-β locus [60]	Amitriptyline: partially reversible global DNA hypomethylation [53] Escitalopram: reversed hypermethylation at P11 [61]	—	Valproic acid: affects DNA methylation through upregulation of HDACs and MeCP2 [59]
Transcription	—	—	Atomoxetine: alters mRNA and protein doses of NET and NMDA receptor subunits 1, A, and 2B [37] D-amphetamine: increases synaptic protein expression spinophilin in the striatum [38], upregulates spinophilin and VGLUT1 in thalamus [39]	—
miRNA	Antipsychotics and mood stabilizers: increase miR-134 levels without reaching control levels in patients treated for bipolar disorder [18]	Prolonged fluoxetine (SERT inhibitor): increases miR-16 levels in serotonergic raphe nuclei, which in turn reduce SERT expression; upon infusion, fluoxetine leads to release of the neurotrophic factor S100b, which acts on noradrenergic cells of the locus coeruleus by decreasing miR-16, thus turning on the expression of SERT in noradrenergic neurons [18]	—	Lithium: consistently changed expression of miR-34a, miR-152, miR-155, and miR-221 in LCLs from bipolar vs. control subjects [62]; 31 miRNAs have been found to be modified in treatment with valproic acid in the rat hippocampus [18]

Abbreviations: HDAC, histone deacetylase; LCL, lymphoblastoid cell lines; MAO, monoamine oxidase; NMDA, N-methyl-D-aspartate; SERT, serotonin transporter; VGLUT, vesicular glutamate transporter.

gene, implicated in the etiology of depression, was investigated and imipramine was shown to cause hyperacetylation of H3 at *BDNF* promoters. Amitriptyline increases H3 acetylation by inhibiting histone deacetylase (HDAC) activity [49]. Fluoxetine decreased acetylation of H3 in the caudate putamen, frontal cortex, and hippocampus. In stressed mice, reduced H3 acetylation in the hippocampus was reversed by fluoxetine [50]. Valproic acid, one of the most studied psychotropics with respect to epigenetic mechanisms, has been reported to increase levels of acetylated histone H3 and H4, promoting gene expression associated with hippocampal neurogenesis [45]. Valproic acid targets the *RELN* gene, which is thought to play a role in bipolar disorder and schizophrenia. It has been shown to induce expression of reelin [46,47].

DNA methylation

Antipsychotics also influence DNA methylation. Haloperidol causes decreased global DNA methylation levels [58]. Clozapine reduced cytosine methylation at the Gadd45-β locus [60]. Of the antidepressants, amitriptyline has been shown to induce partially reversible global DNA hypomethylation [53]. Escitalopram reverses hypermethylation at P11. Because expression of P11 is associated with depression, this action may contribute to the efficacy of this drug [61]. Fluoxetine reverses decreased histone H3K9 trimethylation in the hippocampus [56]. The monoamine oxidase (MAO) inhibitors have been shown to demethylate dimethylated H3K4 by breaking down lysine-specific demethylase 1 (LSD1), inhibiting transcription. This indicates that selective LSD1 inhibitors might be effective in the treatment of depression [54,55]. Kim et al. [59] showed that valproic acid affects DNA methylation through upregulation of HDACs and MeCP2. Valproic acid has also been observed to cause demethylation of the reelin promoter [48] and global demethylation of CpG in astrocytes of the cortex and hippocampus [53].

miRNAs

As shown in Table 20.3, antipsychotics, antidepressants, and mood stabilizers are all reported to affect miRNAs.

Adverse effects of psychotropic drugs

Psychotropic drug-induced adverse effects may also result from epigenetic effects. Using a cell line from human islets where the expression of insulin is modulated by an inducible form of the bHLH transcription factor E47, phenothiazines were found to increase the expression of the insulin gene promoter acutely but suppressed expression with chronic exposure. This provides a mechanism for the clinical emergence of diabetes in patients treated with these drugs over time [64]. Microarray assessment of gene expression in mouse frontal cortex identified downregulation of genes involved in adenosine triphosphate (ATP) biosynthesis and lipid metabolism following chronic treatment with stimulants and increased expression patterns of genes encoding signaling molecules such as insulin that are involved in control of food intake with chronic neuroleptic treatment [65].

Abnormal involuntary movements are a concern in short- and long-term use of neuroleptics. Haloperidol has been reported to induce expression of transcription factor nuclear factor-kappaB

(NF-κB), which is composed of 50 and 65 kDA subunits. Haloperidol but not clozapine was found to increase apomorphine-induced stereotyped behavior that was associated with a decrease in striatal DAT expression in rats. Haloperidol did not change nigral expression of the p65 subunit, but clozapine decreased it, and both induced significant decreases in nigral expression of NF-κB p50, suggesting that a decrease in nigral expression of the p50 subunit may increase vulnerability of the dopaminergic neurons to the neurotoxic effect of the p65 subunit in haloperidol-treated animals [66]. Haloperidol and clozapine treatment over 30 days were found to significantly decrease glutamate transporter 1 (GLT1) expression; however, haloperidol produced more consistent effects, manifested primarily in the dorsal striatum and suggesting that deficiency in glutamate transport may potentially play a role in the motor side effects of typical neuroleptics [67]. Using nerve growth factor-inducible B (NGFIB) knockout mice, haloperidol-induced increases in striatal enkephalin mRNA were totally abolished and neurotensin mRNA expression reduced. Catalepsy induced by raclopride, a specific dopamine (D2/D3) antagonist, was completely abolished; the cataleptic response to SEM 23390, a dopamine (D1) agonist, was preserved [68]. Animals treated chronically with haloperidol but not clozapine showed a regionally selective increase in area density of FosB immunoreactive neurons in the dorsolateral and ventrolateral central pretectal nucleus (CPN) and in both the core shells of the nucleus accumbens (NAc). Only animals receiving chronic haloperidol showed vacuous chewing movements, the animal equivalent of tardive dyskinesia in humans, implicating these brain regions with these motor side effects [69].

Investigating gene expression is also being used as a tool to better understand adverse effects of ADHD medications. An example is atomoxetine, which was associated with mild but significant increases in heart rate and blood pressure and mild QT prolongation [70]. *hERG* (human ether-a-go-go related gene) encodes the α subunit of a potassium ion channel protein (I_{kr}) that mediates the repolarization current of the cardiac action potential [71], and high atomoxetine concentrations (not in therapeutic ranges) have been shown to result in significant reductions of *hERG* expression [72]. This suggests a potentially greater risk in subjects with overdoses, in those with slower metabolism due to cytochrome P450 (CYP) *2D6* enzyme variants and impaired hepatic and renal clearance [72], or for a combination of medications, as has been reported in two case reports [73,74]. Haloperidol has been reported to induce Fos expression in the nucleus accumbens and dorsal striatum [75], increase proapoptotic Bcl-XS [76], and induce expression of transcription factor NF-κB, thereby increasing vulnerability to neurotoxic effects [66]. It also causes expression of NGFIB, thereby inducing catalepsy [68]. Interestingly, pretreatment with atypical antipsychotics has also been shown to have neuroprotective actions by attenuating neurotoxicity of beta-amyloid by modulating Bax and Bcl-X(l/s) expression [76].

Immediate early genes

Immediate early genes (IEGs) such as c-*fos* and *Homer 1a* are activated by numerous cellular stimuli including drugs. Studies in animals are providing clues to the brain areas activated by acute and chronic administration of various psychotropic drugs, helping to identify regions of interest for psychopathology, as well as drug-related adverse effects. As reviewed by Elia et al. [77], acute administration of methylphenidate and amphetamine has been shown to increase expression of c-*fos* and *Homer 1a* in corticostriatal circuits implicated in attentional functions. Methylphenidate resulted in a greater number of cortical cells expressing c-*fos* than amphetamine, suggesting that the dopaminergic

mesoneocortical system may be more sensitive to methylphenidate than amphetamine [78]. In contrast, modafanil induced c-*fos* expression in neurons of the anterior hypothalamic nucleus and adjacent suprachiasmatic borders, suggesting that many areas may be involved in maintaining alertness [78]. IEG expression may also help differentiate the different brain areas affected by the various neuroleptics. In cynomolgus monkeys, haloperidol was reported to induce Fos in nucleus accumbens shell and dorsal striatum, whereas clozapine induced expression primarily in the nucleus accumbens and islands of Calleja [79,80]. Clozapine may actively antagonize the dopamine D2 receptor blockade on striatal fos expression [75]. Acute, but not chronic, treatment with typical neuroleptics was reported to induce c-*fos* mRNA in the dorsolateral striatum, while all neuroleptics induced c-*fos* mRNA in the shell region of the nucleus acumens with acute and chronic administration. Because there is no noted tolerance to neuroleptics it is hypothesized that the nucleus accumbens may be the critical site for the antipsychotic effect [79].

Conclusion

The relevance of epigenetics in child psychiatry continues to gain momentum. Genetic studies stratifying samples based on environmental risk exposure may increase the chance of identifying susceptibility genes. Furthermore, epigenetics may also potentially be helpful in differentiating between diseases, as many of the same common and rare genetic variants have been implicated in most psychiatric disorders. For example, a postmortem brain study identified marked upregulation of hippocampal apoptosis genes in patients with bipolar disorder and downregulation in patients with schizophrenia not related to medication exposure. Differentiating the two groups was the downregulation of antioxidant genes in bipolar but not schizophrenic patients [81]. Advances in understanding epigenetic configuration and mechanisms in normal brains will be essential in the interpretation of findings in psychiatric studies. Research in this area is difficult. Complexities abound given our limited knowledge of normal brain development and the fact that neither brain cells and networks nor the environment remain static. Hence, prospective longitudinal studies, rather than brief snapshots in time, may be necessary, as individual variability to susceptibility or resilience also plays a role.

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Glossary

Amphetamine, atomoxetine, methylphenidate Drugs used to treat attention deficit hyperactivity disorder.

CHAPTER

Epigenetic Mechanisms of Drug Addiction Vulnerability

21

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KEY CONCEPTS

- Drug addiction is characterized by persistent alterations in gene expression throughout the limbic circuitry of the brain.
- Drug use, as well as other environmental factors that enhance vulnerability to addiction (e.g., stress), yield similar chromatin 'landscapes' within the brain's reward circuitry.
- Acute drug use is associated with an immediate increase in euchromatic transcriptional control (reflected by increased levels of histone phosphorylation, acetylation and transcriptionally permissive methylation), particularly at immediate early genes in the nucleus accumbens (NAc). Homeostasis is quickly maintained by counteracting alterations in the chromatin state (e.g., increased repressive methylation).
- Chronic drug use is associated with persistent euchromatic regulation (e.g., heightened levels of histone acetylation, decreased histone methylation at immediate early genes and heterochromatic loci, and increased expression of plasticity-associated genes (e.g., BDNF) leading to increased dendritic spine density and glutamatergic input to the NAc.
- Behavioral responses associated with chronic drug use can be directly manipulated through the modulation of chromatin modifying/associated proteins.
- Chronic stress (both early in life and in adulthood), as well as previous drug use, cross-sensitize animals to the rewarding, reinforcing and psychomotor activating effects of subsequent drug use.
- Drug-induced epigenetic changes are potentially heritable.

ABBREVIATIONS

5mC 5-Methylcytosine
ACF1 ATP-utilizing chromatin assembly and remodeling factor 1
AVP Arginine vasopressin
BDNF Brain-derived neurotrophic factor gene
BLA Basolateral amygdala
BRG1 Brahma-related gene 1
CaMKIIα Calcium-dependent kinase IIα gene
CBP CREB-binding protein
Cdk5 Cyclin-dependent kinase-5 gene
ChIP Chromatin immunoprecipitation
CPP Conditioned place preference
CPu Caudate putamen
CREB cAMP response element-binding protein
CRF Corticotropin releasing factor
DARPP-32 Dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kDa
DNMT DNA methyltransferase
EE Environmental enrichment
ELS Early-life stress
FACS Fluorescence-activated cell sorting
$GABA_A$ gamma-Aminobutyric acid A
H3K9me2 Dimethylation at lysine 9 on histone H3
H3K9me3 Trimethylation at lysine 9 on histone H3
HAT Histone acetyltransferase
HDAC Histone deacetylase
HDACi HDAC inhibitors
HMT Histone methyltransferase
HPA axis Hypothalamic–pituitary–adrenal axis
LINE-1 Long-interspersed nuclear element 1
LTP Long-term potentiation
MAPK Mitogen-activated protein kinase
MDMA 3,4-Methylenedioxy methamphetamine
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
mPFC Medial prefrontal cortex
MSK1 Mitogen- and stress-activated protein kinase 1
MSN Medium spiny neuron
NAc Nucleus accumbens
PP1 Protein phosphatase 1
PTMs Posttranslational modifications
SAHA Suberoylanilide hydroxamic acid
SIRT1 Sirtuin 1 (NAD-dependent deacetylase sirtuin 1)
SWI/SNF SWItch/Sucrose NonFermentable
TET1 Ten–eleven translocation 1
VTA Ventral tegmental area
WSTF Williams syndrome transcription factor

Introduction

Prior to Watson and Crick's monumental discovery of the DNA double helix, scientists had speculated that not all genes were active in a cell at a given time. Conrad Waddington used the term epigenetics to describe the molecular mechanisms that translate a single genotype into a variety of phenotypic outputs [1]. Today, epigenetics typically refers to altered gene expression patterns that arise from changes in chromatin structure, events initiated by signaling cascades that relay information from the extracellular environment. Gene expression patterns that are regulated epigenetically, through such mechanisms as DNA methylation, histone posttranslational modifications (PTMs), and nucleosome positioning can be both dynamic and stable throughout the lifetime of an organism, and in some cases, potentially heritable to their offspring.

In recent years, dysregulation of gene expression through epigenetic mechanisms has been uncovered as a driving force behind many human diseases, including cancer, neurodevelopmental, and autoimmune disorders. It is becoming increasingly clear that a variety of epigenetic mechanisms influence numerous aspects of neural plasticity in the brain, including memory formation, stress responses, cognition, and maladaptive responses to drugs of abuse. In the case of drug addiction, genetic factors represent approximately 40–60% of an individual's risk of becoming an addict [2]. This leaves roughly 50% of the risk under the influence of alternative factors, including early-life stress, adulthood anxiety and depression, previous abuse of the same or different illicit substances, gestational drug exposure, and transgenerational inheritance, all of which are capable of modifying gene expression patterns, in part, through chromatin-based mechanisms.

In this chapter, we will discuss the epigenetic mechanisms that underlie the addicted state and individual vulnerabilities to addiction. Drug addiction itself can be described as a chronic relapsing disorder characterized by maladaptive neural plasticity and pathological behaviors of drug seeking and use in spite of adverse consequences. Interestingly, many drugs of abuse, as well as stress and mood disorders, share common neural and molecular pathways, particularly the brain's reward circuitry (the mesolimbic dopamine system), which includes dopaminergic neurons in the ventral tegmental area (VTA) of the midbrain and their postsynaptic targets in the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc). The NAc also receives numerous projections from glutamatergic afferents arising from the mPFC, basolateral amygdala (BLA), and ventral subiculum of the hippocampus, all of which are known to influence affective behaviors (Figure 21.1). These brain regions have been shown to be essential for drug-induced changes in conditioned reward. Evidence now suggests that epigenetic mechanisms of gene regulation in these regions play a multifaceted role in priming vulnerability to addiction, driving acute behavioral responses to drugs of abuse, and establishing the life-long effects of drug use that can persist even following periods of physical and affective withdrawal. A more thorough analysis of the epigenetic mechanisms governing gene transcription in these common neural pathways is crucial for our understanding of drug addiction, as well as other disorders that utilize these pathways.

Epigenetic regulation of gene expression

Epigenetic modes of gene regulation can be grouped into five general areas: histone PTMs, chromatin remodeling, and variant exchange, as well as DNA methylation and regulation by non-coding

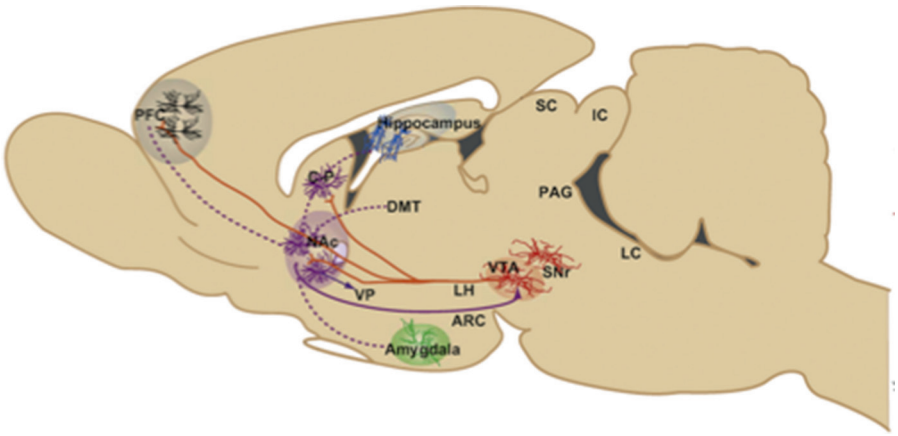

FIGURE 21.1

Circuit anatomy of major cell types underlying addiction-related plasticity. VTA dopamine projection neurons project directly onto nucleus NAc and mPFC neurons, as well as onto amygdala and hippocampal neurons (the latter projections are not shown in the figure). GABAergic afferents (some direct, some indirect) from the NAc to the VTA provide feedback to VTA dopamine neurons. Glutamatergic projections to the NAc from mPFC, amygdala, and hippocampus release glutamate onto medium spiny neuron (MSN) synapses. Each structure contains specialized neuronal cell types that play distinct roles in addiction-associated behaviors. The major cell types, include amygdala and NAc spiny neurons, PFC and hippocampal CA3 pyramidal neurons, and VTA dopamine neurons. Numerous drugs of abuse aberrantly influence the structure of each of these cell types.

Reproduced with permission from Russo SJ, et al. The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. Trends Neurosci 2010; 33: 267–76; Russo SJ, et al. Neurotrophic factors and structural plasticity in addiction. Neuropharmacology 2009; 56(Suppl 1): 73–82; Maze I, Russo SJ. Transcriptional mechanisms: underlying addiction-related structural plasticity. Mol Interv 2010; **10***: 219–30.*

RNAs. Although individually important, these mechanisms often work together to orchestrate precise phenotypic outputs in mammalian cells. Epigenetic mechanisms of gene expression are discussed in Chapter 2 of this book.

Epigenetic mechanisms in addiction

In spite of having diverse mechanisms of acute action, the downstream effects of drugs of abuse converge on the brain's reward circuitry, the mesolimbic dopamine system. Both acute and chronic drug use provokes changes in gene expression in brain regions associated with this pathway. Additionally, many of the brain regions that project to or receive projections from the mesolimbic dopamine system, including the amygdala, hippocampus, and hypothalamus, are known to be aberrantly regulated by exposure to drugs of abuse. All of these circuits play important roles in reward-based and emotional memory, lending to the notion that drug addiction involves the formation and maintenance of powerful emotional memories.

Histone and DNA modifications are emerging as key epigenetic mechanisms through which drugs of abuse prime, desensitize, or otherwise alter gene expression patterns to bring about synaptic, dendritic and behavioral changes associated with the addicted state. Current research suggests that drugs of abuse tend to induce an overall euchromatic chromatin state; however, gene expression profiles and epigenetic landscapes associated with initial drug exposures appear to significantly differ from those observed following repeated drug administration, as well as following periods of abstinence and withdrawal.

Acute drug exposure: rapid and specific epigenetic responses

Although acute drug treatment paradigms in rodents often involve only a single exposure to a drug of abuse, in humans acute drug experiences may define more casual or initial encounters with a particular abused substance that do not result in behavioral states associated with addiction (e.g., pathological drug seeking and taking). Interestingly, it is now known that even a single exposure to cocaine, for example, can induce an enhancement in euchromatic chromosomal structure leading to increased and defined patterns of gene expression in neurons located within the brain's reward circuitry (e.g., NAc). Pronounced epigenetic changes include an increase in the magnitude of H3 and H4 acetylation at immediate early genes [3], increased H3K9me2 at synaptic genes [4], and increased heterochromatic H3K9me3 at intergenic genomic loci [5,6]. These changes are believed to facilitate rapid and targeted alterations in the expression of numerous genes involved in synaptic plasticity.

Histone acetylation

Acute exposure to psychostimulants, such as cocaine and amphetamine, increases acetylation of histones H3 and H4 in the NAc and dorsal striatum (caudate putamen, CPu). Histone acetylation on H3 at positions K9/14 and on H4 at positions K5/8/12/16 are markers of transcriptional permissiveness and represent the majority of chromatin-associated histone acetylation sites studied in response to acute drug exposure. Although the acetylation of genes (often at promoters) may not lead to enhanced transcription, work by Renthal et al. [7] demonstrated a clear correlation between differentially expressed genes and histone acetylation at promoter regulatory sites following cocaine. It should be noted, however, that not all highly expressed genes show increases in histone acetylation in response to cocaine. For example, genes encoding constitutively expressed proteins, such as β-tubulin, tyrosinehydroxylase, and histone H4, remained unaffected by both acute and chronic cocaine exposure, indicating a potentially high level of specificity for drug-induced chromatin changes [3]. Genes that are heavily acetylated following acute cocaine exposure, especially in the case of histone H4, include immediate early genes, such as *fosB* and c-*fos*. Additionally, it was found that genomic regions displaying cocaine-induced increases in acetyl H3 and H4 rarely overlap, suggesting distinct mechanisms of gene regulation through independent histone PTMs.

Attempts to identify the specific histone acetyltransferases (HATs) and histone deacetylases (HDACs) involved in drug-induced molecular and behavioral responses have been complicated by the promiscuity of HDAC inhibitors and the assembly of multisubunit HDAC complexes. For example, there is substantial evidence that class I HDACs (e.g., HDAC1, 2, 3, and 8) interact with class IIa HDACs (e.g., HDAC4, 5, and 9) *in vivo* to form multisubunit transcriptional repressor complexes. Additionally, the HDAC inhibitors (HDACi) sodium butyrate, valproate, and suberoylanilide hydroxamic acid (SAHA) were originally thought to non-specifically block class I and II HDACs, but have recently been shown to potently and selectively target class I HDACs more specifically [8].

Early work using these HDACi strongly indicated an important role for HDACs in modulating drug-induced behavioral responses. Astoundingly, intra-NAc infusion of sodium butyrate during initial cocaine use was found to enhance animals' behavioral responses to the drug [3,9]. Similarly, infusion of dopamine D1-specific agonists also enhances behavioral responses to many drugs of abuse [10]. Because most abused substances increase synaptic dopamine to concentrations that activate D1-like receptors, it seems plausible that drugs of abuse induce targeted changes in histone acetylation through selective signaling-mediated HDAC inhibition or HAT activation. Consistent with this hypothesis, intra-NAc delivery of sodium butyrate along with D1 agonists further enhanced cocaine responsiveness over that of HDACi alone [10]. When HDACi were infused prior to cocaine exposure or during periods of cocaine intake, they were found to attenuate behavioral responses to cocaine, while concomitant treatment of the inhibitor with cocaine dramatically enhanced behavioral responses [11–13]. These findings were further delineated through conditional knockout of HDAC1, 2, or 3, as well as through use of an HDACi with improved specificity, revealing HDAC1 as a key regulator of initial cocaine responsiveness [13]. Kennedy et al. [13] also demonstrated that acute pharmacological inhibition of HDAC1 enhanced cocaine sensitivity, consistent with previous findings using sodium butyrate. It will be interesting in the future to determine if, and through which mechanisms (e.g., PTM, translocations), HDAC1 activity is suppressed in response to cocaine. Although work from Kennedy et al. suggested HDAC1 as the critical class I HDAC involved in the development of cocaine-induced psychomotor sensitization, work from the laboratory of Wood [14,15] has further indicated HDAC3 as a key mediator in the development of cocaine-context-associated memories and extinction to cocaine-conditioned place preference. These data suggest that specific HDACs, even of the same class, contribute differently to an animal's behavioral responsiveness to cocaine.

Histone acetyltransferases have also been shown to play significant roles in acute drug responses. For example, mice genetically depleted of the HAT CREB-binding protein (CBP) exhibit reduced sensitivity to cocaine and reduced acetylation at the *fosB* promoter [16]. Complete loss of CBP in NAc neurons has similarly been shown to significantly alter both acetylation at the c-*fos* promoter, as well as its expression, in response to cocaine [17]. Taken together, these results indicate a powerful role for the "writers" and "erasers" of histone acetylation in the molecular and behavioral effects of initial drug exposure; however, much more work is needed to better understand the contributions of these molecular phenomena to the drug-addicted state.

Histone phosphorylation

Although much less is known about the role of histone phosphorylation in addiction, cocaine administration has been shown to rapidly induce H3 phosphorylation (e.g., H3S10phos) and phosphoacetylation (H3S10phos/K14ac) in the striatum [3,18]. These phosphorylation events are generally mediated by activation of the mitogen-activated protein kinase (MAPK) signaling cascade, and more specifically by the H3 kinase MSK1. Depletion of MSK1 was shown to reduce locomotor responses to cocaine, although it is currently unclear whether such attenuation of psychomotor sensitization is a direct result of MSK1's activity on H3. In addition, D1 receptor-expressing medium spiny neurons in the striatum have been shown to exhibit increased phosphorylation and phosphoacetylation following a single injection of cocaine [6,19]. Furthermore, the protein phosphatase 1 inhibitor DARPP-32 has been demonstrated to regulate histone phosphorylation in the striatum in response to cocaine exposure [18]. Because phosphorylation is a common PTM utilized in signaling cascades, it is likely that the

phosphorylation of histones and chromatin-modifying enzymes may serve as an early instructive signal in response to initial drug exposure.

Histone methylation

Like histone acetylation, initial exposure to drugs of abuse can induce rapid and persistent changes in histone methylation. Two important histone methylation signatures that have been identified to change in response to cocaine include euchromatic dimethylation of H3K9 and heterochromatic trimethylation of H3K9, both of which are increased in expression by acute cocaine exposure and are repressive in nature. Current data suggest that cocaine-induced increases in histone acetylation enable improved access and binding of histone methyltransferases (HMTs) to chromatin, and such increases may function to homeostatically suppress euchromatin formation. More recent histone PTM studies examining FACS-sorted nuclei from D1 compared with D2 medium spiny neurons in the striatum of cocaine-naive, compared with acutely treated, animals has helped to shed light on methylation responses occurring within these molecularly distinct neuronal subtypes [6]. In their study, Jordi et al. [6] demonstrated that D2 neurons exhibit a dramatic, but transient, increase in mono-, di-, and trimethyl H3K9 following a single injection of cocaine, while D1 neurons show persistent increases in H3K9me2 and H3K9me3, as well as moderate increases in H3K4 methylation states 24 hours after a single exposure to cocaine. Considering these differences in histone methylation between neuronal subtypes, it will be important in future studies to avoid potentially confusing results by studying epigenetic processes in distinct neuronal subpopulations.

Chromatin remodeling

Little is known regarding the effects of acute drug use on chromatin remodeling complex function, though the cocaine-induced expansion of euchromatin is likely to be aided by chromatin remodeling activities. Given the potential for diverse biological functions arising from subunit switching in these multiprotein complexes, it is possible that specific combinations of chromatin remodeler subunits, perhaps with even greater variation in function as the result of PTMs, may play distinct roles in chromatin alterations following acute drug exposure. Chromatin remodeling complexes contain many histone-binding domains, such as bromodomains and chromodomains. It is therefore likely that drug-induced increases in histone acetylation and methylation will be accompanied by alterations in chromatin remodeler binding events.

There is also limited evidence to suggest that MAPK signaling can regulate chromatin remodeler activity in other cell types. In fibroblasts, for example, the catalytic subunit of the mammalian SWI/SNF remodeler BRG1 forms a complex with MSK1 to induce nucleosomal remodeling at the promoters of immediate early genes [20]. Following promoter phosphorylation by MSK1, the SWI/SNF complex remodels nucleosomes locally to enable the binding of transcription factors and other components of the transcriptional machinery. A more comprehensive understanding of chromatin remodeler function in the context of drug addiction is clearly warranted.

DNA methylation

Although little is known about the function of DNA methylation in shaping molecular and behavioral responses to acute drug use, the expression of both maintenance and *de novo* DNA

methyltransferases, DNMT1 and DNMT3a/b, respectively, in the NAc are reduced in expression following acute cocaine treatment [21,22]. Pharmacological inhibition and viral-mediated knockdown of DNMT3a in the NAc have also been shown to enhance behavioral responses to cocaine. These findings indicate a role for DNMT3a, but not necessarily for DNA methylation itself, in the early stages of drug addiction. The methylation status of DNA is difficult to study, and most current methods cannot distinguish between the reduced and oxidized forms of this modification, each of which may function differently in response to drugs of abuse. An important future step in this area will be to improve characterizations of genome-wide DNA methylation, with the ability to distinguish between reduced and oxidized forms of cytosine methylation.

Non-coding RNAs

Although it is highly likely that non-coding RNAs play a role in shaping the chromatin landscape during initial responses to drugs of abuse, nothing is currently known; however, specific microRNAs (miRNAs) have been shown to participate in epigenetic responses to chronic drug exposure and will be discussed later in this chapter.

Chronic drug use

The effects of chronic drug use on the epigenetic landscape are fairly distinct from those of acute drug exposure, although some epigenetic signatures are known to persist. Following chronic drug use, acetylation appears to plateau at genes initially targeted by the drug, and further increases in acetylation by HDAC inhibitors begin to disrupt or oppose drug-induced behavioral responses [13]. The genomic targets of acetylation also appear to change, resulting in the transcription of many genes previously implicated in long-lasting forms of neuronal plasticity (e.g., long-term potentiation, LTP). Another defining characteristic of chronic drug use is the global repression of heterochromatic histone methylation. This de-repression of genomic targets leads to increases in dendritic spines (sites of glutamatergic input), thereby sensitizing neurons to further periods of stimulation. Taken together, chronic drug use produces a distinct epigenetic landscape that appears to be hyperacetylated, hypomethylated, and perhaps structurally unstable.

Histone acetylation

Increases in the levels of histone acetylation observed at the initiation of drug exposure appear to be maintained throughout chronic drug use, although the targets of acetylation shift from immediate early genes to genes that are involved in long-term plasticity including *BDNF* (brain-derived neurotrophic factor), *Cdk5* (cyclin-dependent kinase-5), and *CaMKIIα* (calcium-dependent kinase IIα) [3,23,24]. Also, a shift in histone acetylation from a predominantly H4 acetylated state to an H3 acetylated state has been observed [7]. Chronic drug administration also likely engages different HAT and HDAC activities than those observed following a single drug exposure. As mentioned earlier, class I HDAC inhibition appears to strongly enhance behavioral responses to acute cocaine when administered concurrently. However, chronic class I HDAC inhibition, coupled with chronic cocaine treatment, results in diminished behavioral responses to the drug. This happens, in part, through an increase in $GABA_A$ expression in NAc neurons and subsequent enhanced inhibitory tone.

HDAC4 activity is also thought to be suppressed by chronic cocaine administration. When overexpressed in the NAc, HDAC4 has been shown to attenuate H3K9/K14ac levels and block conditioned place preference (CPP) in rodents [3]. Additionally, when infused into the NAc shell, HDAC4 partially attenuates cocaine self-administration, an etiologically relevant rodent model of human drug addiction. HDAC4 has also recently been shown to be vitally important for synaptic development and plasticity, with its genomic targets in the brain including *CBP*, *BDNF II* and *III*, *fosB*, *Cdk5*, *CaMKIIα*, *Gria2*, *Grin2a*, and *Grin2b* [23,25]. Several of these HDAC4 gene targets are also acetylated after acute cocaine treatment, while others are involved in forms of long-term potentiation and long-term depression. Thus, HDAC4 suppression may function in maintaining the epigenetic landscape established by acute cocaine administration and also may play a role in long-term changes associated with neural plasticity. The expression of class III HDACs, such as SIRT1 and SIRT2, has also been shown to be induced in response to chronic cocaine. Specifically, the promoters of these genes become acetylated by chronic drug treatment [7], and upon closer inspection, were found to be regulated by the stable splice product of the *fosB* gene product, ΔFosB. Both pharmacological inhibition and knockdown of SIRT1 and SIRT2 attenuate cocaine-induced behavioral responses, including self-administration and conditioned reward (e.g., CPP); however, overexpression of SIRT1 and SIRT2 exerts the opposite effect. These data indicate that SIRT1and SIRT2 HDAC activity may prune acetylation to promote drug-addicted phenotypes. Interestingly, another class IIa HDAC, HDAC5, has been shown to be phosphorylated in a *CaMKIIα*-dependent manner by repeated cocaine exposure leading to its nuclear export and the hyperacetylation of target genes in the NAc [9,26]. Through various knockdown and mutational studies, these endogenous effects of chronic cocaine administration on HDAC5 function have been demonstrated to result in increased behavioral responsivity to cocaine.

Histone methylation

Arguably one of the most important epigenetic regulatory mechanisms mediating chronic cocaine-induced behaviors is the antagonistic relationship between the transcription factor ΔFosB and the H3K9 dimethyltransferase G9a. As mentioned, H4ac and H3K9me2 are two histone PTMs found to be enriched at the *fosB* promoter during acute drug treatment. Here, H3K9me2 attenuates cocaine-induced expression of *fosB* and its splice product, ΔFosB, but does not blunt it completely. Eventually, through repeated exposure to a drug of abuse—in this case, cocaine—sufficient amounts of ΔFosB are produced, and it accumulates in the striatum [4]. The accumulation of ΔFosB during repeated cocaine administration results in G9a repression and reduced global levels of H3K9me2 [4]. Even without cocaine present, overexpression of ΔFosB in the NAc is sufficient to reduce G9a recruitment to its target promoters. Furthermore, reductions observed in G9a activity enhance behavioral responses to cocaine and increase dendritic spine plasticity in the NAc [4]. This change in synaptic architecture is a hallmark of long-term plasticity, and generally correlates with enhanced behavioral sensitivity to psychostimulants [27,28]. Intriguingly, this increase in dendritic spine density, as induced through chronic cocaine, is capable of being rescued by G9a overexpression in the NAc [4]. Similarly, inhibition of ΔFosB, which results in a reinstatement of G9a activity, blocks the ability of chronic cocaine to increase dendritic spine density. These results elucidate an important and long-lasting epigenetic mechanism underlying altered neural plasticity and behavior associated with repeated drug use (Figure 21.2). Another curious finding was that H3K9me3, a repressive mark normally associated with heterochromatin domains and repetitive genomic sequences, is

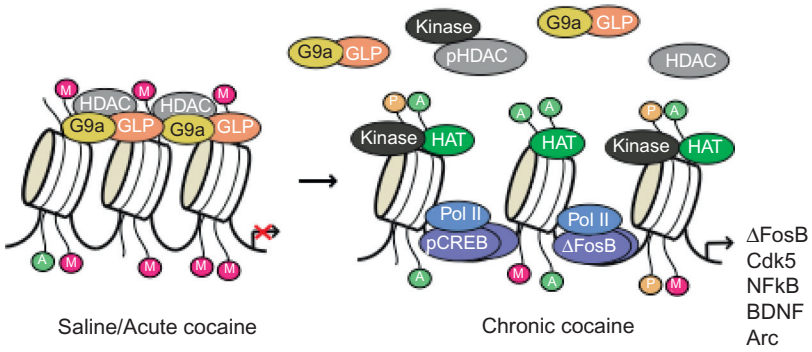

FIGURE 21.2

Chronic cocaine administration increases transcriptional plasticity in the NAc through chromatin-dependent mechanisms. Methylation of H3K9 promotes chromatin compaction and gene repression. Following either saline or acute cocaine exposure, a multimeric complex of HMTs, primarily G9a and GLP, along with specific HDACs (e.g., HDAC5), bind to chromatin and repress transcription. Following repeated cocaine administration, however, repressive HDAC and HMT binding to plasticity-related promoters is reduced, which, in combination with heightened kinase activity and binding of HATs, ΔFosB, pCREB, and RNA polymerase II (Pol II), results in active transcription. Such decreased repressive chromatin states result in increased structural plasticity on NAc MSNs.

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reduced in striatal neurons after chronic cocaine exposure and displays decreased genomic enrichment throughout intergenic loci in the NAc [5]. Commensurate with decreases in H3K9me3 binding at heterochromatic loci was an increase in repetitive element unsilencing, including the expression of long-interspersed nuclear element 1 (LINE-1). These elements have been shown to promote genomic instability; however, the specific roles of these elements in drug addiction remain to be elucidated.

Chromatin remodeling

Once again, very little is known regarding the specific roles of chromatin remodelers in drug addiction. Recent evidence suggests that the expression levels of specific chromatin remodeling enzymes and associated subunits, including brahma-related gene 1 (BRG1), ATP-utilizing chromatin assembly and remodeling factor 1 (ACF1), and Williams syndrome transcription factor (WSTF), are dynamically regulated by chronic cocaine and opiate [29,30] exposure. It will be necessary in future studies to determine the extent of this regulation, as well as whether these remodelers are capable of influencing addiction-related phenotypes.

DNA methylation

Given the relative stability of DNA methylation, this mode of epigenetic regulation appears as a likely candidate in explaining the persistent effects of chronic drug use. Unfortunately, few experiments

have been performed to directly assess the impact of manipulating DNA methylation in animal models of drug abuse, in part due to the lack of tools available to screen genome-wide DNA methylation patterns.

In 1984, Francis Crick proposed that DNA methylation might serve as a memory mechanism at the molecular level. Since then, it has been shown that general inhibitors of DNMT activity alter DNA methylation both globally in the brain and more specifically at neural plasticity-associated genes, such as *reelin* and *BDNF*. Studies of *de novo* DNMT3a during hippocampal-dependent fear-conditioned learning have revealed rapid methylation responses following periods of learning, followed by transcriptional silencing of the memory suppressor protein phosphatase 1 (PP1) and demethylation/transcriptional activation of *BDNF* and reelin [31,32]. These results imply that DNA methylation and demethylation may be dynamically regulated in the brain.

In addiction models, local infusion of the DNMT inhibitors RG108 or zebularine have been shown to increase behavioral responses to chronic cocaine administration in a manner similar to HDAC inhibition and ΔFosB-mediated repression of H3K9me2. Unlike cocaine's effects on histone methylation, DNMT3a-mediated gene repression was found to increase dendritic spine plasticity on NAc neurons [22]. These data indicate that the role of DNMTs, and perhaps DNA methylation itself, following chronic cocaine exposure may not simply be to repress plasticity-related gene expression, as would have been predicted. Along this line, previous studies have revealed instances (see above) where DNA methylation, particularly in gene bodies, can be activating. Another mechanism by which DNMTs may promote dendritic plasticity is through their relationship with histone acetylation. In several studies, inhibition of DNMT activity has been found to block histone acetylation, as well as memory formation. This indicates that the activities and/or presence of DNMTs on chromatin may help to facilitate histone acetylation, perhaps through the formation of functional DNMT–histone "writer"/"eraser" complexes. In addition, expression of methyl-CpG-binding protein 2 (MeCP2) has been found to increase after chronic cocaine exposure in the dorsal striatum [33]. Knockdown of MeCP2 in NAc appears to attenuate cocaine administration [34]. Conversely, amphetamine reward was shown to be enhanced by MeCP2 knockdown in the dorsal striatum [35], revealing multiple roles for this protein in drug addiction. Another possible mechanism through which DNA methylation might function in shaping the epigenetic landscape is through its oxidation (hydroxy-, formyl-, carboxymethylcytosine). Preliminary work by Nestler and colleagues has revealed that chronic cocaine decreases TET1 expression, an enzyme that catalyzes 5mC oxidation, in the NAc. This decrease in TET1 expression was also found to potentiate behavioral responses to cocaine, an effect that may be linked to TET1's recently discovered role in memory formation. These results underscore the important interplay between multiple forms of epigenetic regulation during the transition from acute/casual drug use to compulsive/pathological drug taking.

Non-coding RNAs

Following chronic cocaine administration, alterations in the expression of several miRNAs have been observed in the rat striatum. Next-generation sequencing recently identified tens of miRNAs that appear to be altered in NAc following chronic cocaine exposure. Of these, miR-181a was shown to be increased in its expression, and miR-124 and let-7d were shown to be decreased in their expression [36–40]. Furthermore, artificially enhancing these differences resulted in a corresponding enhancement of cocaine reward. Another miRNA that opposes addictive-like behaviors in rodents is miR-212,

which inhibits cocaine intake in rats after chronic administration of the drug [41]. The mechanisms of miR-212's action appear, in part, to be mediated through its indirect activation of the transcription factor CREB, which has been shown to antagonize cocaine reward [42]. These results demonstrate that non-coding RNAs likely play a significant role in mediating epigenetic responses to chronic drug use; however, much more work is needed in this area.

Vulnerability to drug addiction

A question that has yet to be fully addressed in the field of drug addiction, particularly with regard to the epigenetic regulation underlying this disease, is what makes some individuals more vulnerable than others to becoming drug addicted. As mentioned previously, in spite of having nearly identical genomes, some inbred laboratory animals develop compulsive drug-taking behaviors during periods of chronic drug exposure, while others limit drug intake or refuse drugs all together. The question is why. One possibility is that epigenetic landscapes established early in life in response to environmental factors are responsible for these differences, perhaps helping to explain the other ~50% of vulnerable individuals who are not genetically predisposed to engage in such behaviors. Do environmental factors known to enhance vulnerability to addiction, including early-life stress, mood disorders, previous exposure to the same or different drugs of abuse, and perhaps even parental drug exposure prior to and/or during gestation, establish an "epigenetic" domino effect that leads to addiction? Or do drugs of abuse and these various environmental factors simply share common neuronal pathways and molecular targets? Although little is known, this next section will attempt to shed light on these potentially related phenomena in the context of epigenetic plasticity.

Early-life stress

Early-life stress (ELS) can be subdivided into childhood maltreatment (neglect; physical, sexual, and psychological abuse) and stressful life events. The degree of risk for adult psychopathology tends to correlate with the severity of childhood maltreatment and the number of stressful life events. Of those who have experienced childhood maltreatment, a random U.S. sampling revealed that 22% of men and 20% of women report physical abuse, 14% of men and 32% of women report sexual abuse, and 21% report having experienced both [43]. Another study, performed among 1362 individuals from six Native American tribes, found that four or more childhood maltreatment experiences resulted in a threefold greater risk of becoming drug dependent in men, while the same number of maltreatments resulted in a sevenfold greater risk in women [44]. Additionally, a survey of women in drug rehabilitation centers found that 75% of women in treatment programs reported having been sexually abused [45].

In the brain, ELS is characterized by a life-long elevation in glucocorticoid secretion, heightened endocrine responsiveness to subsequent stressors, and disruption of the hypothalamic–pituitary–adrenal (HPA) axis, all of which are considered to be pathogenic factors in mood and cognitive disorders. Emerging evidence suggests that ELS in rats, by maternal separation or handling, results in significant and persistent effects on the mesolimbic dopamine pathway, the same pathway targeted by drugs of abuse. In fact, it was found that rats that experience ELS display dose-dependent increases

in cocaine-induced locomotor activity, as well as heightened levels of anxiety [46]. Epigenetic mechanisms, including DNA methylation, are now being studied in the context of ELS. Murgatroyd and colleagues [46] have found that ELS decreases DNA methylation in the hypothalamus to stably induce the expression of arginine vasopressin (AVP), a stress hormone that triggers neuroendocrine and behavioral alterations that are characteristic of clinical depression. These changes in AVP expression persisted for at least one year, and they found that *CaMKII*-dependent phosphorylation of MeCP2 prevents its binding to the *Avp* enhancer, resulting in its increased expression. Other studies indicate the ELS differentially regulates BDNF expression in multiple brain regions. In the ventral tegmental area (VTA), for example, sustained upregulation of BDNF is observed after ELS. Additionally, decreased BDNF expression is observed in the hippocampus following ELS [47], and Roth et al. [48] reported that maltreatment in rats produces altered BDNF expression in the adult prefrontal cortex. Given its differential regulation, it is plausible that BDNF signaling may help instruct epigenetic changes in these brain regions.

Early-life stress paradigms in rodents, specifically maternal separation, have demonstrated that ELS induces a rapid increase in histone H4 acetylation at synaptic plasticity genes, including *Arc* and *Egr1*, and increases dendritic length in CA3 hippocampal neurons [49]. This increase in histone H4 acetylation is reminiscent of H4 acetylation at the *fosB* promoter in the NAc observed following acute cocaine exposure. Another research group recently found that ELS represses HDAC1, 3, 7, 8, and 10 activity in the forebrain neocortex with corresponding increases in H4 acetylation at K12, events that are sustained through adulthood [50]. In spite of strong correlations between ELS and drug addiction in humans, the epigenetic relationship between ELS and addiction is still very unclear, due, in part, to the fact that many drug addiction studies focus on epigenetic changes in the striatum, while most ELS models focus almost exclusively on molecular alterations occurring throughout the HPA axis and in the hippocampus.

Mood disorders

High rates of comorbidity between mood and substance abuse disorders has led to increasing interest in uncovering possible epigenetic links between the two. Additionally, chronic stress in rodents is known to cross-sensitize animals to subsequent drugs of abuse. Acute and chronic stress can promote both anxiety and depression in humans, in part, through the regulation of levels of histone acetylation and methylation. In turn, stress alters the corticotropin releasing factor (CRF) system, BDNF levels, and the activity of transcription factors (e.g., CREB) in the brain. In the hippocampus, for example, levels of the repressive heterochromatic mark, H3K9me3, increase dramatically from one "acute" to seven "chronic" days of restraint stress [51]. Furthermore, the active methylation mark H3K4me3 is reduced following chronic stress, particularly in CA1 [51]. Chronic stress is known to increase the rewarding properties of cocaine through altered CRF and phospho-CREB signaling across several brain regions [52]. Chronic social defeat stress, an etiologically valid rodent model of human depression, is commonly used to study anxiety and depressive-like symptoms at the levels of brain circuitry, signaling networks and epigenomic function. Similar to chronic cocaine, chronic social defeat stress induces an increase in NAc BDNF in susceptible animals (i.e., those that exhibit a depressive-like phenotype), with a similar increase in NAc BDNF observed in depressed humans [53,54]. This corresponds to a period of genomic de-repression, as decreased HMT activity

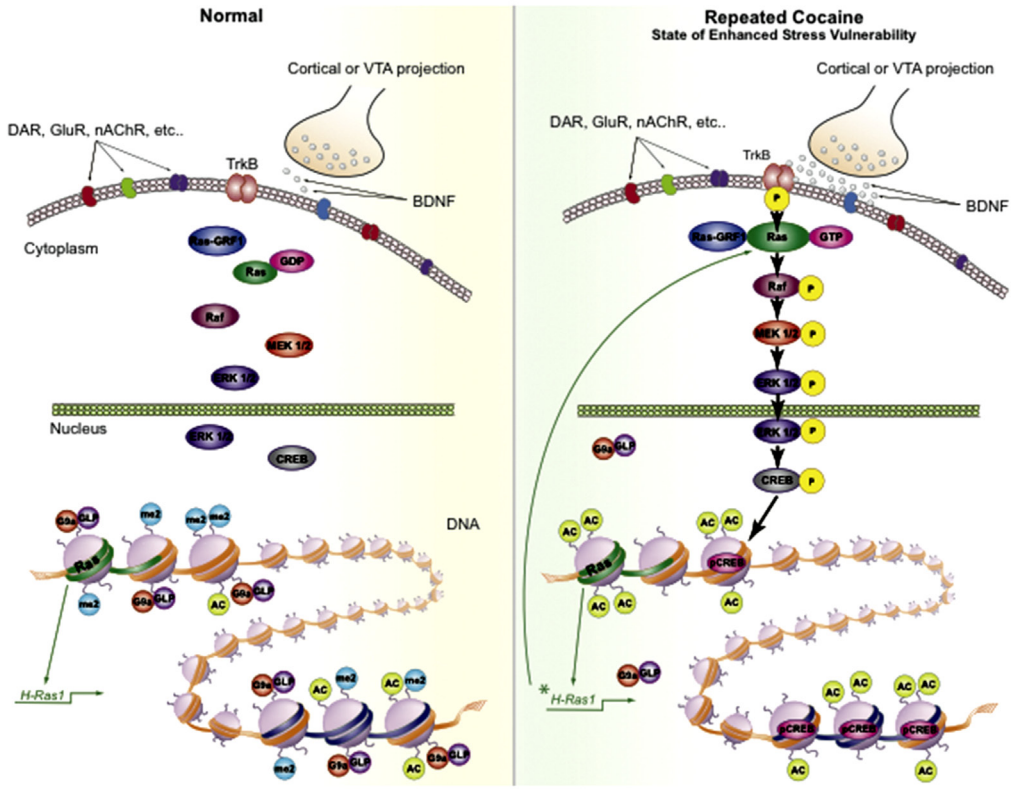

FIGURE 21.3

Increased vulnerability to stress following cocaine-induced enhancement of BDNF signaling in the NAc. Chronic cocaine administration promotes vulnerability to the depressive-like effects of social defeat stress by priming BDNF signaling in the NAc. Under control conditions, BDNF activation of TrkB signaling is homeostatic. After repeated cocaine, however, BDNF–TrkB signaling is increased in the NAc, resulting in enhanced phosphorylation and activity of downstream signaling mediators including CREB. This cocaine-initiated adaptive response occurs through increased Ras expression due to decreased G9a binding at the *H-Ras1* gene. Ras is also a target for CREB, thereby creating a positive feed-forward loop initiating CREB activation and Ras expression, as well as depression-associated behaviors.

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(specifically, G9a, GLP, and SUV39H1) and reduced CoREST are observed in the NAc of susceptible animals [55]. Corresponding decreases in H3K9me2 in susceptible animals are also reminiscent of the chromatin state observed following chronic cocaine exposure [4]. Interestingly, recent findings have demonstrated that BDNF overexpression in the VTA potentiates social defeat stress-induced cross-sensitization to amphetamine through increases in ΔFosB expression [56]. Finally, increased numbers of dendritic spines on NAc medium spiny neurons have been observed following

chronic social defeat stress in susceptible animals [57], similar to that observed with chronic cocaine administration. In a recent study that directly investigated the relationship between stress and drug addiction, mice were repeatedly administered cocaine and then exposed to a subthreshold level of stress—one that does not induce social avoidance or anhedonia in mice. Surprisingly, animals that were chronically treated with cocaine showed increased vulnerability to stressful episodes and exhibited depressive-like behaviors. This vulnerability was demonstrated to result, in part, from decreased G9a activity in the NAc and subsequent loss of H3K9 methylation, a phenotype that is common to both stress and chronic drug exposure. Remarkably, forced G9a expression in susceptible mice was found to restore normal behavioral responses to subthreshold levels of stress, an effect associated with decreased BDNF signaling (Figure 21.3). Therefore, future therapeutic drugs aimed at enhancing G9a activity in depressed and drug-addicted individuals may represent a possible form of therapy for both afflictions.

Environmental enrichment

Countering maladaptive and detrimental forms of adulthood and early-life stress, animals may also experience extended periods of enrichment and positive reinforcement, which often result in more positive forms of adaptive plasticity (e.g., enhanced learning, reduced responses to stress). Experiences that are environmentally enriching include positive social interactions, occupational enrichment (provide control or challenges), physical enrichment (exercise and play), sensory stimulation, and nutritional enrichment (novel or varied food). Such positive experiences can similarly be modeled in laboratory animals using a robust paradigm known as environmental enrichment (EE). Intriguingly, animals that experience EE display reduced vulnerability to addictive-like behaviors [58,59]; for example, rats raised with EE also show reduced self-administration of cocaine and amphetamine [58,60,61]. In most of these studies, EE was maintained throughout the lifetime of the animal. However, Nader and colleagues [62] showed that changing an animal from its standard housing to an EE in adulthood was sufficient to increase cocaine place preference, with associated increases in phospho-CREB expression and reductions in CRF1R and CRF2R in the NAc shell. These results underscore the potency of environmental stress in promoting vulnerability to drug abuse. Although currently understudied, it will be interesting in the future to determine which, if any, features of the epigenetic landscape resulting from life-long EE promote an animal's resilience to future life stressors and/or drug exposures.

Prior drug exposure

Prior exposure to the same or different drugs of abuse has also been shown to enhance vulnerability to drug addiction. For example, repeated Δ^9-tetrahydrocannabinol induces behavioral sensitization to either the same cannabinoid or morphine administered after a 2-week period of abstinence [63]. Cocaine and alcohol are also known to behaviorally cross-sensitize through mutual upregulation of striatal dopamine transporter binding sites [64]. A more recent study found that previous use of 3,4-methylenedioxy methamphetamine (MDMA, or "ecstacy") and amphetamine cross-sensitize to one another [65]. Regardless of the combination of drugs, cross-sensitization tends to occur, supporting the hypothesis that drugs of abuse converge onto common molecular pathways in the brain

and likely induce similar epigenetic outcomes. A recent example of this was the finding that chronic morphine exposure, which acts through distinct pharmacological mechanisms from those of cocaine, similarly reduces the expression and activity of G9a and results in enhanced morphine reward and sensitization [66]. Such reductions in repressive histone methylation in NAc, however, were further demonstrated to result in a dampening of naloxone-precipitated morphine withdrawal, indicating potentially distinct functions for G9a in mediating the physical, compared with affective, withdrawal symptoms elicited by these two drugs.

Gestational drug exposure

Drug use during pregnancy is not recommended. Indeed, a quick check of WebMD promptly warns, "Using drugs—legal or illegal—during pregnancy has a direct impact on the fetus." Aside from the risk of overdose, gestational exposure to cocaine can lead to significant deficits in a child's cognitive performance, information processing, and attention to tasks later in life. As with postnatal drug sensitization and cross-sensitization, prenatal drug exposure modestly enhances substance abuse vulnerability. For example, prenatal exposure to nicotine through cigarette smoking has been shown to increase DNA methylation within the *BDNF-6* exon, which subsequently downregulates its expression [67]. Additionally, cocaine use in pregnant female mice has been shown to globally decrease DNA methylation patterns in the offspring [68]. Most epigenetic changes resulting from gestational drug exposure remain elusive; however, the expansion of chromatin studies in the context of developmental biology may help to shed light on this important topic.

Epigenetic inheritance of addiction

Although it is difficult to rule out confounding non-gametic inheritance, there is some evidence that epigenetic modifications on chromatin may be heritable through the germ line. Because females are born with all of their ova, environmental effects on males, who continually produce sperm following puberty, are now the focus of many investigations into the transgenerational inheritance of phenotypic outcomes. Indeed, reliable transmission of physiological and behavioral traits has been observed through the paternal lineage. Whereas approximately 96–99% of histones are replaced with protamines during spermatogenesis, a small number of histone modifications remain in mature sperm, such as H3K4me2/3 and H3K27me3. These histone PTMs are found to specifically mark certain developmental loci, non-coding RNAs, imprinted genes, and bivalent promoters. In a recent study of drug-induced transgenerational inheritance [69], male rats that had self-administered cocaine for extended periods of time were allowed to breed with cocaine-naive female rats, and histone acetylation was assessed. Interestingly, the male, but not female, offspring of cocaine self-administering sires appeared to inherit a cocaine-resistance phenotype, which included increased association of acetyl H3 with *BDNF* promoters in the medial prefrontal cortex and diminished cocaine self-administration. Additionally, acetylation of H3 at the *BDNF* promoter in sperm of male offspring was found to be altered. These results indicate that voluntary intake of cocaine in fathers can result in epigenetic reprogramming of the germ line, with subsequent effects on gene expression in the adult brain, as well as resistance to cocaine reinforcement that may persist across generations.

Future directions

Although significant progress has been made in recent years in improving our understanding of the epigenetic landscape of addiction, many questions and challenges still remain. One of the greatest challenges to the emerging field of neuroepigenetics is the difficulty in obtaining sufficient starting material for genome-wide and biochemical studies. Improving the sensitivity of tools used to assess genome-wide enrichment patterns of histone PTMs, transcription factors, etc. (e.g., ChIP-seq, RNA-seq, DNA methylation assays) will be paramount for moving forward. Much of the work in the brain is also complicated by the fact that neural tissues consist of a variety of neuronal and non-neuronal cellular subtypes. Recent work by Jordi and colleagues [6] addressed this problem using cell-sorting technologies coupled to fluorescence to identify histone marks that are enriched in D1 compared with D2 dopamine neurons in the striatum. However, the precise genomic location of these marks has yet to be determined using this method. More work also needs to be done to better elucidate the intracellular signaling pathways that are involved in instructing histone modification states and DNA methylation patterns in neurons. It is likely that intracellular signaling events help to target these epigenetic modifications to specific genes, possibly through interactions with chromatin remodelers, histone modifying enzymes, and/or specific histone PTMs at target genes; however, very little is known with regard to upstream mechanisms controlling these chromatin-associated transcriptional outputs.

Given the emerging importance of non-coding RNAs and chromatin remodeling proteins, it is surprising that so little is known about how these epigenetic regulators function in psychiatric disease. Work showing that long non-coding RNAs are able to tether and target chromatin remodeling complexes to specific genomic loci demonstrates a potentially important role for these RNAs in orchestrating chromatin structural changes observed in response to drugs of abuse. Similarly, miRNAs have been found to promote transdifferentiation in fibroblasts and are known to modify drug responses in neurons. A more thorough analysis of their regulation and function in drug addiction is necessary.

Perhaps most importantly, the mechanistic relationships between drug addiction and environmental factors known to enhance vulnerability to addiction must be further explored. Research suggests that many of the environmental factors that predispose individuals to becoming addicted target similar brain regions, signaling networks and transcriptional machinery. For example, both chronic stress in "susceptible" animals and chronic cocaine use are known to reduce the expression of G9a, while restoration of G9a attenuates susceptibility to both stress and drug addiction. Moreover, stress and drug addiction cross-sensitize to one another, and, again, restoration of G9a levels reduces this effect [55]. Given the strong correlation between environmental factors and drug addiction, it will be critically important to identify common features of each epigenetic landscape and to utilize this information to develop treatment strategies that prevent disease initiation and reinstatement.

Conclusions

The current goal of neuroepigenetics research in the field of drug addiction is to better understand how even a single drug exposure can induce and sustain long-lasting changes in gene expression. At its most basic level, epigenetic studies also shed light on how an individual's life experiences establish stable changes in gene expression, which influence not only that individual's risk for addiction but also their risk in a wide variety of psychiatric diseases. The hope of epigenetic research in this and

other fields is to identify gene products and regulatory mechanisms that will serve as future targets for therapeutic intervention. Epigenetic signatures in peripheral tissues may also be uncovered to help diagnose addiction vulnerability and/or precise stages of addiction, providing a valuable means of customized options for future prevention and treatment using pharmacotherapy. In these ways, epigenetic research has the potential to yield unprecedented advances in our understanding, diagnosis, and treatment of drug addiction.

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Glossary

Acute drug use In humans, often refers to casual but not pathological drug use; in rodents, usually refers to a single exposure to a drug of abuse.

Bromodomain Protein domain that binds acetylated histones.

Chromatin Nuclear material that makes up the chromosome. Consists of DNA; histones H1, H2A, H2B, H3, and H4 (or variants thereof); and other chromatin-associated proteins.

Chromatin remodeling complexes Multimeric protein complexes (often containing numerous subunits) that utilize ATP to alter nucleosome positioning along the DNA template.

Chromodomain Protein domain that binds methylated histones.

Chronic drug use In humans, refers to pathological, compulsive drug use; in rodents, usually consists of at least 7 days of experimenter-delivered drug exposure or extended periods of drug self-administration.

Chronic social defeat stress Etiologically relevant rodent model of human depression involving chronic exposure to aggression, both physical and sensory. Such stress results in either susceptible (exhibiting symptoms of depression, such as reduced social interaction, anhedonia, increased HPA-axis engagement, etc.) or resilient animals.

Cocaine place preference (CPP) Form of Pavlovian conditioning used to measure motivational effects of objects or experiences.

De novo Addition of a new chemical modification where none existed before.

DNA methyltransferase (DNMT) Enzymes that catalyze the addition of methyl groups to the 5′ carbon positions of cytosine nucleotides within DNA.

Dopaminergic Dopamine neuromodulator-releasing neurons.

Drug addiction Pathological drug seeking and use in spite of adverse physiological, psychological, and social consequences.

Early-life stress Early-life maltreatment (e.g., neglect; physical, sexual, or psychological abuse) or stress; often modeled in rodents by preweaning maternal separation or chronic handling.

Euchromatin Transcriptionally active chromatin that is less condensed.

Glutaminergic Glutamate (excitatory) neurotransmitter-releasing neurons.

Heterochromatin Transcriptionally silent chromatin that is highly compact.

Long non-coding RNA (lncRNA) Non-protein-coding RNA transcripts longer than 200 nucleotides that may facilitate multiprotein complex docking on DNA.

Maintenance Replication of an existing chemical modification (e.g., DNA methylation).

Methyl-CpG-binding protein Proteins containing specific binding domains that allow for direct interactions with methylated DNA.

microRNA Non-protein-coding RNA transcripts that are approximately 22 nucleotides in length that base pair with mRNA transcripts to regulate protein expression.

Nucleosome The basic unit of transcription; 147 bp of DNA wrapped ~1.7 times around an octamer of histones (two copies each of H2A, H2B, H3, and H4).

Psychostimulants Psychoactive drugs such as cocaine, amphetamine, or methylphenidate that stimulate the central nervous system by boosting the release of specific neurotransmitters and neuromodulators (e.g., dopamine).

Self-administration paradigm (rodents) Etiologically relevant model of human drug addiction, where animals undergo operant conditioning to receive intravenous drug injections. Many variations of this behavioral paradigm exist.

CHAPTER

Mitoepigenetics and Neuropsychiatric Disorders

22

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KEY CONCEPT

- Mitoepigenetics refers to an epigenetic system involving bidirectional communication between the nucleus and mitochondria; this chapter discusses the possible role of mitoepigenetics in neuropsychiatric disorders.

ABBREVIATIONS

5hmC 5-Hydroxymethylcytosine
5-LOX 5-Lipoxygenase
5mC 5-Methylcytosine
AD Alzheimer's disease
ALS Amyotrophic lateral sclerosis
CNS Central nervous system
CpG Cytosine–phosphate–guanine
DNMT DNA methyltransferase
HDAC Histone deacetylase
MELAS Myopathy, encephalopathy, lactic acidosis, stroke-like episodes
MERRF Myoclonus epilepsy with ragged red fibers

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mitosRNAs Mitochondrial genome-encoded small RNAs
mtDNA Mitochondrial DNA
mtDNMT1 Mitochondrial DNMT1
ND1 NADH dehydrogenase subunit 1
ND6 NADH dehydrogenase subunit 6
nDNA Nuclear DNA
OXPHOS Oxidative phosphorylation
POLG mtDNA polymerase γ
PolgA mtDNA polymerase γ catalytic subunit
SAM *S*-adenosylmethionine
T-DMRs Tissue-dependent differentially methylated regions
TET1 Ten–eleven translocation 1
VPA Valproic acid

Mitoepigenetics overview and relevance for brain disorders

The term mitoepigenetics was first introduced in association with a complex epigenetics-based interplay between the nucleus and mitochondria [1]. In this context, it was noted that mitochondria, intracellular organelles with their own mitochondrial DNA (mtDNA), influence the epigenetic machinery operative in the nucleus and in nuclear DNA (nDNA) and that epigenetic modifications occur within these organelles including mtDNA [2].

According to popular belief (i.e., the theory of endosymbiosis), the origin of mitochondria, which share ultrastructural similarities with bacterial cells and contain their own mtDNA inside eukaryotic cells (that contain a separate and different nDNA), rests with evolutionary forces [3]. Thus, during evolution, bacterial cells became engulfed and modified to become eukaryotic mitochondria. In addition to being different and physically separated from the nuclear genome, the mitochondrial genome is inherited solely from the mother; in mammals, the paternal mitochondria are specifically destroyed by proteolysis in early embryonic development [4]. The mammalian mitochondrial genome encompasses a relatively small (16,295–16,826 bp), circular, double-stranded mtDNA that contains only 37 genes, of which 13 encode polypeptides/proteins. Furthermore, mtDNA encodes two rRNAs critical for the translation of the 13 protein-encoding mRNAs. These 13 proteins are members of the oxidative phosphorylation (OXPHOS) complexes. All other mitochondrial proteins (thousands), including the additional members of the OXPHOS complexes as well as some components of epigenetic machinery, are encoded in nDNA (i.e., by the so-called mitochondrial genes). These proteins are imported from the cytoplasm via mitochondria targeting sequences as well as by alternative mechanisms. The exact mitochondrial composition and content of these imported proteins is tissue and cell-type specific [5]. Furthermore, a cell may contain hundreds of mitochondria whose numbers and morphology continuously change, and each mitochondrion may contain multiple copies of mtDNA.

In the nucleus, a peculiar morphological combination of nDNA and nuclear proteins, particularly histones, gives rise to a functionally important structure called chromatin. Modifications of this structure by epigenetic mechanisms affecting both the nuclear proteins, such as histone modifications, and nDNA, such as DNA methylation to generate 5-methylcytosine (5mC) and DNA hydroxymethylation to generate 5-hydroxymethylcytosine (5hmC), are at the heart of nuclear epigenetics. Because of the belief that mitochondria do not contain histones, although recent data suggest that mtDNA-binding histones exist inside the mitochondria [6], and because of the under-representation of the CpG dinucleotide sequence in mammalian mtDNA [7], the possibility of intramitochondrial epigenetic modifications has generally been dismissed [2]. Nevertheless, the structure of mtDNA appears to be impacted by a number of intra-mitochondrial proteins that help create a mitochondrial chromatin-like structure

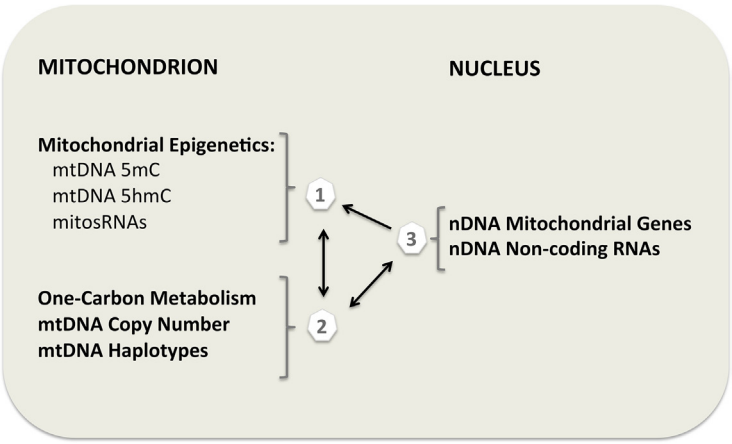

FIGURE 22.1

The elements of mitoepigenetics.

called nucleoid [8]. Nucleoids are anchored to the inner mitochondrial membrane, and it has been proposed that, similar to nuclear epigenetics, the mitochondrial epigenetic mechanisms could be involved in nucleoid modifications [2]. Moreover, recent evidence suggests that, similar to nDNA, mtDNA is also susceptible to 5mC and 5hmC modifications [1].

Because mitochondria are endowed with the capacity for biogenesis even in postmitotic cells such as neurons, a close interplay between the two genomes, nDNA and mtDNA, is required for the maintenance of cell physiology. The optimal tissue-specific and function-dependent amount of cellular mitochondria is maintained by both the nuclear signaling-mediated nDNA and mtDNA transcription and by the retrograde signaling from the mitochondria to the nucleus [9]. A similar system of bidirectional communication between the nucleus and mitochondria exists with respect to epigenetic processes and has been termed mitoepigenetics [1].

Figure 22.1 shows the key elements of mitoepigenetics. First, mtDNA is subject to epigenetic marking by cytosine methylation and hydroxymethylation via processes collectively termed mitochondrial epigenetics, which include the nDNA-encoded mitochondrial components of epigenetic machinery. Second, mitochondrial activity, mtDNA copy number, and mtDNA haplotypes modulate the methylation pattern in nDNA. Third, nuclear epigenetic mechanisms that regulate the expression of nDNA-encoded genes influence mitochondrial physiology directly by modifying the expression of a number of nuclear-encoded mitochondrial genes.

The relevance of mitochondrial physiology and pathobiology for brain functioning and the occurrence and progression of neuropsychiatric disorders has long been suspected. A recent review of the field concluded that, although most patients with major psychiatric disorders do not have classic mitochondrial disorders, an impaired mitochondrial function may affect key cellular processes, thereby altering synaptic functioning and contributing to the deteriorating long-term course of these illnesses [10]. Particularly evident is the key role of mtDNA integrity in health. Hundreds of diseases are caused by mtDNA mutations [11–13]. In normal cells, multiple copies of intracellular mtDNA

are identical (homoplasmy), in contrast to cells harboring mtDNA mutations. In the latter case, mutations usually affect some but not all mtDNAs within a cell, causing heteroplasmy. Ultimately, the proportion of normal and mutated mtDNA in different tissues determines the clinical pathology. Many of the primary mtDNA-related diseases are characterized by pathological alterations in the central nervous system (CNS), for example, syndromes such as MELAS (myopathy, encephalopathy, lactic acidosis, stroke-like episodes) and MERRF (myoclonus epilepsy with ragged red fibers). Other CNS disorders frequently linked to mitochondrial dysfunction include a group of neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS) [14] and the pathologies associated with old age such as Parkinson's disease [15] and Alzheimer's disease (AD) [16,17]. The association of mitochondrial pathologies with old age typically rests on the mitochondrial free-radical theory of aging, which proposes that aging is driven by mtDNA damage and mutations caused by the production of reactive oxygen species [18]. However, in the brain, aging appears to lead not only to changes in nDNA hydroxymethylation [19] but also to brain region-specific alterations in the 5hmC content in mtDNA [20]. With respect to major psychiatric disorders, including schizophrenia and bipolar disorder, recent evidence suggests the involvement of mtDNA haplotypes and mitochondria-related nuclear gene polymorphisms [21–23]. In addition, drugs, including the compounds used for treatment of neuropsychiatric disorders, can cause off-target mitochondrial effects leading to mitochondrial and mtDNA toxicity [24]. One such example is the anticonvulsant and mood stabilizer valproic acid (VPA), a drug that also acts as a histone deacetylase (HDAC) inhibitor and epigenetic modifier. VPA has been shown to be capable of altering the expression of ten–eleven translocation 1 (TET1), an enzyme involved in 5hmC formation, and consequently changing the hydroxymethylation of mtDNA [25]. Interestingly, VPA is known to be fatal for individuals with some mitochondrial diseases [26].

Collectively, these studies indicate a profound involvement of mitochondrial physiology in brain functioning and in brain-related pathobiological processes. The contribution of neuroepigenetics to the field of neuropsychiatric disorders has been mainly through the study of nuclear epigenetics. It appears that not only do mitochondria influence this type of epigenetics but also epigenetic mechanisms are operative within mitochondria and as a basis of bidirectional communications between the nucleus and mitochondria. This chapter summarizes the current state of CNS-related mitoepigenetic research and points to emerging future directions.

Mitochondrial epigenetics

In contrast to the term mitoepigenetics, which includes the above-noted bidirectional epigenetic interactions between mitochondria and nuclei, the term mitochondrial epigenetics is restricted to molecular events dealing solely with intramitochondrial epigenetics and the modifications of mitochondrial genome [1,2]. The first evidence for epigenetic modifications of mtDNA (i.e., the presence of 5mC in mammalian mtDNA) dates back to two reports submitted for publication in 1973 [27,28]. These initial findings of mammalian mtDNA methylation were generally disregarded until they were rediscovered in 2011 [29,30], along with the first evidence for the presence of 5hmC in mammalian mtDNA [20,29]. Also recent is the observation that mitochondria are the site of epigenetic mechanisms involving mitochondrial non-coding RNAs [31,32]. Currently, the field of mitochondrial epigenetics has generated sufficient evidence to justify additional research efforts aimed at understanding the functional relevance of epigenetic mitochondrial modifications and their possible significance as health- and disease-related biomarkers.

mtDNA methylation

Several forms of DNA methyltransferases (DNMTs) critical for DNA cytosine methylation have recently been found in mammalian mitochondria. Because mtDNA does not encode DNMT genes, these nuclear-encoded proteins have to be imported into the mitochondria in order to generate 5mC in mtDNA sequences. There are multiple import pathways used for protein entry into the mitochondria, and they form a network capable of importing a diverse range of substrates [33]. Some of the imported proteins possess a mitochondrial targeting sequence, but others do not. Of the known DNMTs, only a small portion of DNMT1 mRNA is translated from an upstream ATG start codon that contains a mitochondrial import signal to generate a DNMT1 protein equipped with the mitochondrial targeting sequence—mitochondrial DNMT1 (mtDNMT1) [29]. Consequently, DNMT1 immunolabeling was documented in mitochondria [20,29,34]. Furthermore, it was shown [29] that upon mtDNMT1 mRNA expression, the translated mtDNMT1 protein, which contains both the DNMT1 catalytic domain and the mitochondrial targeting sequence, is imported into the mitochondria, where it is present in the mitochondrial matrix, bound to mtDNA. These authors concluded that the interactions of mtDNMT1 with mtDNA are CpG dependent and particularly evident in the D-loop control region. In spite of the lack of mitochondrial targeting sequences in other DNMTs, it was reported that in addition to DNMT1, mitochondria also contain DNMT3A [30,35] and DNMT3B [34]. Hence, it is possible that all three enzymes play a role in determining mtDNA 5mC pattern and levels. In this scenario, all three DNMT subtypes (i.e., mtDNMT1, DNMT3A, and DNMT3B) would catalyze the synthesis of 5mC from cytosine. In addition, DNMT3A and DNMT3B might be able to impact the location of cytosines available for methylation—for example, by mtDNMT1. Namely, the mammalian DNMT3A and DNMT3B, but not DNMT1, are redox-dependent DNA dehydroxymethylases capable of converting 5hmC to cytosine [36]. Such a direct action of DNMT3A and DNMT3B on 5hmC, if operative in mitochondria, would be particularly significant considering the high abundance and wide distribution of 5hmC in mtDNA [37].

The presence and content of 5mC in mammalian mtDNA have been evaluated by a variety of techniques. A direct visualization of mtDNA 5mC content in CNS was achieved by immunofluorescence methodologies [30,35]. Furthermore, mtDNA 5mC content and distribution were measured by immunoprecipitation [29] and by using enzyme-linked immunosorbent assays [20]. In the latter approach, it is essential that mitochondria are extracted and purified prior to mtDNA extraction [20]. The same purification step would be preferable in other 5mC assays of mtDNA, although often the sequencing strategies are applied to total DNA extracts, which might account for technical difficulties in measuring the mtDNA-specific 5mC levels [38]. Furthermore, compared with ncDNA, mtDNA lacks the typical CpG islands and generally has fewer CpG dinucleotides [39], which in ncDNA are considered to be the primary targets for epigenetic marking. Nevertheless, bisulfite sequencing of human and mouse mtDNA established the presence of 5mC in the control D-loop and revealed that methylated residues are preferentially located in non-CpG nucleotides [34]. The presence of 5mC in the D-loop of human mtDNA was confirmed by bisulfite pyrosequencing, which also revealed 5mC in two additional mtDNA regions: the transfer RNA phenylalanine (MT-TF) and 12 S ribosomal RNA (MT-RNR1) gene [40]. The latter two sites of mtDNA methylation appear to be susceptible to the action of airborne pollutants, and 5mC presence in these sites may serve as a biomarker and possibly play a functional role in cell response to environmental stimuli [40]. Furthermore, it was found that in human blood cells the methylation status of mtDNA at the 12 S ribosomal gene decreases during aging [41].

Aging of mammalian organisms appears to be associated with significant changes in DNA methylation and hydroxymethylation. Epigenetic marks, such as 5mC and 5hmC and numerous histone modifications, are emerging as important factors of the overall variation in life expectancy that are responsible for the establishment of specific expression programs [42], which in turn may influence the development and progression of aging-associated illnesses. It has been hypothesized that a global decrease of DNA methylation may be a key element or mechanism of aging [43]. However, recent findings suggest that patterns of DNA methylation in CNS gene promoters of imprinted genes are stable over time and that the multiple documented changes with age are likely to involve exceptions to this pattern, possibly associated with specific cellular responses to age-related changes [44]. Similarly, aging did not affect the global hippocampal 5mC content but increased the content of global nDNA 5hmC as well as the 5hmC content in the sequence of the inflammatory gene 5-lipoxygenase (5-LOX) [19]. 5-LOX expression in the brain is upregulated in aging-associated AD [45], and this neurodegenerative disease appears to be linked to a reduction of DNA methylation at the 5-LOX gene promoter [46]. Interestingly, nDNA but not mtDNA hydroxymethylation and methylation are increased in the brain of AD patients compared with normal controls [47].

On the other hand, both mtDNA and nDNA 5mC contents are significantly increased in ALS postmortem samples and in mouse models of this disease [30,35]. These authors found that the ALS-associated motor neuron cell death was linked to increased DNA methylation, and they documented the pathology-associated increase of DNMT3A (and to a lesser extent DNMT1) immunoreactivity in the mitochondria [30]. Furthermore, they were able to obtain experimental neuroprotection with a DNMT inhibitor, RG108. These results demonstrated the feasibility of epigenetic therapy for neurodegenerative pathologies, although it was unclear whether a selective inhibition of mtDNA methylation would be therapeutic in ALS.

mtDNA methylation has recently been implicated in the pathobiology of Down syndrome, the most common human aneuploidy associated with mental retardation and early neurodegeneration. Hence, a study utilizing immortalized lymphoblastoid cells obtained from subjects with Down syndrome found significant reduction of the mitochondrial levels of *S*-adenosylmethionine along with hypomethylation of mtDNA in Down syndrome patients compared with corresponding age-matched controls [48]. In this work, mtDNA methylation was quantified by a method of liquid chromatography–electrospray ionization tandem mass spectrometry.

In the context of neurobiological basis of addiction, it was proposed that CNS effects of ethanol on mitochondrial epigenetics act in concert with nuclear epigenetics to exert cellular and behavioral effects of alcohol [49]. These studies were performed in adult rats exposed chronically to alcohol consumption and in animals after ethanol withdrawal. In the frontal cortex of these rats, ethanol withdrawal, but not chronic ethanol treatment, increased mitochondrial content of DNMT1 protein and global mtDNA 5mC and 5hmC contents. Assays of sequence-specific mtDNA 5mC and 5hmC contents confirmed these results and revealed that even chronic ethanol increased these epigenetic marks in selected mtDNA sequences, albeit to a lesser extent compared with ethanol withdrawal. A possible link between ethanol exposure and mtDNMT1 upregulation and increased mtDNA methylation could involve mechanisms related to oxidative stress [50]. Thus, in studies investigating the relationship between mtDNA methylation and oxidative homeostasis, ethanol was applied to HCT 116 and SH-SY5Y cells. This treatment increased the protein level of mtDNMT1 but not total DNMT1. These investigators concluded that the ethanol-related upregulation of mtDNMT1 is through the relief of p53 suppression [50].

Currently, the available data suggest that mtDNA methylation exists in various tissues, including CNS cells, and that its pattern and extent are modified under physiological conditions and possibly in relation to neuropsychiatric disorders. The main unanswered question is what are the functional consequences of mtDNA methylation at the cellular and organism levels. One possible answer is that mtDNA plays a modulatory role in the regulation of mitochondrial transcription [29,34]. Thus, it was shown that an increase in mtDNMT1 suppressed the expression of NADH dehydrogenase subunit 6 (ND6), the only protein-coding gene on the light strand of mtDNA, while increasing the expression of NADH dehydrogenase subunit 1 (ND1), a protein-coding gene on the heavy strand of mtDNA. The mechanism by which 5mC in mtDNA modifies mitochondrial transcription may reflect a similar mechanism that leads to 5mC-mediated transcription suppression in the nDNA (in the case of ND6 suppression), or the opposite effect on ND1 expression could involve an interaction of the mitochondrial transcription termination factor 1 with 5mC in CpG dinucleotides and/or its interaction with the mtDNA-bound mtDNMT1 protein molecules [2,29]. It is presumed that mtDNA methylation-modified mtDNA transcription would alter mitochondrial transcription in a cell-specific manner that ultimately would change the activity of higher structures such as the CNS.

mtDNA hydroxymethylation

Several scenarios may explain the origin of 5hmC in mtDNA. Similar to nDNA, mtDNA may be subject to enzymatic activity of TET proteins. Various subtypes of TET proteins have been detected in mammalian mitochondria. TET1 and TET2 proteins were found in the mitochondria of mouse neurons grown in primary cultures [20], in mouse 3T3-L1 cell lines [25,34], and in human HeLa cells [34], whereas TET activity was measured in the mitochondrial fraction of human HCT116 cells [51]. Although TET1 immunoreactivity was found in the mitochondria of mouse embryonic stem cells, TET1 knockout did not alter the mtDNA 5hmC content of these cells, suggesting that other TET proteins might be involved in mtDNA hydroxymethylation of mouse embryonic stem cells [51]. Alternatively, 5hmC found in mammalian nDNA (e.g., in the brain) [52,53] and possibly in mtDNA could be the result of oxidative DNA damage, which may account for bases such as 5-hydroxycytosine (5hmC was not measured in these studies). Furthermore, mtDNA hydroxymethylation could occur through covalent addition of a 5-hydroxymethyl group directly to DNA cytosine residues by mtDNMT1 using formaldehyde generated from mitochondrial mixed-function oxidases [29]. Yet another mechanism capable of altering mtDNA 5hmC pattern would involve mitochondrial DNMT3A and DNMT3B, which could affect the mtDNA 5hmC content via their 5hmC dehydroxymethylase activity [36].

A number of methods have been used to assess genome-wide 5hmC distribution. One recently developed assay for a high-resolution mapping of genomic 5hmC is based on a DNA-modification-dependent restriction endonuclease, *Aba*SI, coupled with sequencing (Aba-seq) [37]. Applied to samples obtained from mouse embryonic stem cells, this assay revealed the highest 5hmC site density in the mitochondrial genome in both the CG and CH (H = A, C, or T) context. In mtDNA, normalized CH 5hmC site density was as high as the normalized CG 5hmC site density; in nDNA, the CH 5hmC site density was <1% of the CG 5hmC site density [37].

In the CNS, 5hmC was found in mtDNA extracted from various brain regions of mouse [20], rat [49], and human [47] brain. Aging was associated with brain region-specific alterations in global and sequence-specific mtDNA hydroxymethylation; it decreased mtDNA 5hmC content in the frontal

cortex but not in the cerebellum [20]. This cortical aging-associated 5hmC decrease was found in both CpG and non-CpG regions of mtDNA. Although aging did not affect the 5hmC content of mtDNA extracted from the cerebellum, it did affect mtDNA hydroxymethylation in a subpopulation of cerebellar neurons, the cerebellar Purkinje cells. In contrast to the aging-associated decrease of cortical mtDNA hydroxymethylation, the content of 5hmC was increased in the Purkinje cells of old compared with young mice [20]. In the rat brain, chronic ethanol consumption and ethanol withdrawal increased mtDNA 5hmC content in the frontal cortex (a similar effect was observed with respect to the 5mC content) [49]. These results demonstrated the susceptibility of mtDNA hydroxymethylation to modification in adult brain both during a physiological process (aging) and under the influence of a psychoactive substance (ethanol). Another example of CNS-relevant dynamic mtDNA hydroxymethylation is shown by the ability of the neuropsychiatric drug valproic acid to alter the cell content of 5hmC [25]. The observed plasticity of mtDNA hydroxymethylation and its amenability to pharmacological modulation suggest that altering the content of 5hmC in mtDNA could be targeted for therapeutic purposes. As with mtDNA methylation, the necessary step toward this goal is the elucidation of functional consequences of mtDNA epigenetic marking.

mitosRNAs

Ro et al. [32], who reported that the murine and human mitochondrial genomes encode a great number of small non-coding RNAs, which are predominantly derived from the sense transcripts of the mitochondrial genes, termed these molecules mitochondrial genome-encoded small RNAs (mitosRNAs). In humans, clusters of mitosRNAs show differential tissue-specific contents; for example, they are similar in the brain and heart (organs abundant with postmitotic cells) and different compared to gastrointestinal tissue. These authors suggested that mitosRNAs are the product of currently unidentified mitochondrial ribonucleases. Furthermore, dysregulated mitosRNA expression was generally associated with aberrant mitochondrial gene expression *in vivo* [32]. Although epigenetic research on non-coding RNAs focuses predominantly on nDNA-encoded RNAs, mtDNA-encoded mitosRNAs may be involved in this type of regulation as well [54]. A CNS-relevant physiological mitosRNA mechanism has been inferred by recent experiments with mice. In this study, animals were trained to execute a nose-poke in a port containing various test odors in order to obtain a reward. It was found that in the hippocampus two mitochondrial RNAs (tRNA and Mt-1) gave rise to small non-coding RNAs (25–30 nucleotides). Interestingly, the content of these small RNAs in the brain was specifically increased with training [31]. It is tempting to speculate that mitosRNA-mediated mitochondrial epigenetics could play a role in physiological and pathological memory formation and processing.

Mitochondria and nDNA epigenetics

Mitochondrial dysfunction has been identified as a potent factor in inducing epigenetic changes in the nucleus [55]. Mitochondrial activity is a key player in the interaction among folate metabolism, the methionine cycle, and transmethylation reactions, collectively known as one-carbon metabolism, and is crucial for production of the universal methyl donor *S*-adenosylmethionine (SAM) [56,57]. Hence, by modifying one-carbon metabolism and DNA and protein (e.g., histone) methylation, mitochondria can directly influence nDNA epigenetics (and possibly also mitochondrial epigenetics). Impairments

in one-carbon metabolism, SAM availability, and the consequent pathological methylation processes including aberrant epigenetic DNA methylation have been linked to a number of illnesses, such as neuropsychiatric disorders [56,58–60].

In addition, it was proposed that focusing on haplogroup and individual susceptibility factors in psychiatric disorders by considering mtDNA variants may lead to a better understanding of the pathobiology of neuropsychiatric disorders [21]. With respect to a possible role of mitochondrial variants in regulating nDNA epigenetics, it was discovered that mtDNA haplotypes are capable of modulating the levels of global nDNA methylation and altering the pattern of nuclear gene expression [61]. These researchers showed that isogenic nuclear cybrid cells containing the mtDNA haplogroup J had higher global DNA methylation levels in comparison to other mtDNA haplogroups. Additional studies have confirmed the ability of various mtDNA haplotypes to alter both nuclear gene expression and nDNA methylation [62].

Changes in mtDNA content, usually measured as a ratio between the mitochondrial and the nuclear genome, have been reported in a broad range of human diseases. It was proposed that mtDNA content in body fluids and tissues could be a biomarker of mitochondrial dysfunction [63]. A possible involvement of a variable copy number of neuronal mtDNA in the pathobiology of psychiatric disorders has been suggested for Parkinson's disease and AD [16] and in autism [64]. The latter study compared the activities of mitochondrial electron transport chain complexes and pyruvate dehydrogenase, as well as mtDNA copy number in frontal cortex tissues from autistic and age-matched control subjects. In addition to altered activities of electron transport chain complexes and pyruvate dehydrogenase, autism was associated with a higher mtDNA copy number. Regarding nDNA epigenetics, alterations in cellular mtDNA copy number are associated with altered nDNA methylation; the removal of mtDNA from cells causes both hypomethylation and hypermethylation of nDNA. These changes are reversible, as they can be corrected by the reintroduction of mtDNA [65]. A possible mechanism involved in this interaction between mitochondria and nuclear epigenetics appears to involve the mtDNA copy-number-dependent expression of DNMT1 [66].

Recent experiments in yeast have demonstrated the existence of conserved mitochondria-to-nucleus stress-signaling pathways that regulate aging through epigenetic modulation of nuclear gene expression [67]. This type of epigenetic regulation appears to involve a transient elevation of mitochondrial stress, including mitochondrial reactive oxygen species, and it elicits beneficial responses that extend the lifespan. It remains to be evaluated whether these mechanisms apply to mammalian cells and whether they are relevant for brain aging.

Epigenetics of nDNA-encoded mitochondrial genes

Epigenetic mechanisms that regulate the expression of the nuclear genome also influence mitochondria by modulating the expression of nuclear-encoded mitochondrial genes. As discussed above, mammalian mtDNA encodes only 13 proteins, and all other proteins necessary for mitochondrial structure and functioning are encoded by nuclear mitochondrial genes. Microarray-based DNA methylation analyses of human and murine genomes revealed the existence of numerous tissue-dependent differentially methylated regions (T-DMRs) [68–70]. A recent analysis of 899 nDNA-encoded mitochondrial genes in the brain, heart, and liver tissues identified 636 genes carrying T-DMRs [68]. These authors showed that T-DMRs regulate the expression of nDNA-encoded mitochondrial genes and that hypomethylation of T-DMRs may influence the brain expression of certain transcription factors.

Another example of such epigenetics–mitochondria interaction is the epigenetic regulation of the nuclear-encoded, mtDNA-specific polymerase γ catalytic subunit (*PolgA*) [71]. POLG protein is the only known DNA polymerase that localizes within mitochondria and is essential for mtDNA replication, maintenance, and ultimately affects mtDNA copy number. Kelly et al. [71] identified tissue-specific DNA methylation patterns within the gene body of *PolgA* (i.e., exon 2), which increase as cells differentiate into specialized cell types, and these authors demonstrated a negative correlation between intragenic DNA methylation and the levels of *PolgA* expression, which provided an epigenetic mechanism for regulating mtDNA replication. Yet another mechanism involves nDNA-encoded microRNAs (involved in gene silencing) targeted to mitochondria [72]. A putative role of mitosRNAs in mitoepigenetics has already been discussed. In addition, recent studies have established that mitochondria are one of the destinations of pre- as well as mature microRNAs, and it appears that these nuclear-encoded microRNAs may epigenetically regulate not only nDNA-encoded genes but also transcripts encoded by mtDNA.

Future directions

The relevance of mitoepigenetics for diagnostic and therapeutic modalities in the area of neuropsychiatric illnesses depends on a better understanding of both the mechanisms of mitoepigenetics and the links between altered mitochondrial functioning and the pathobiology of CNS disorders. It appears that mitochondria are gaining attention as a target for developing novel neuropharmacological treatments [10]. Several strategies are being explored for targeting mitochondria with drugs. Targeting mtDNA is one of these strategies—for example, by drugs that interact with POLG and by antiviral drugs [73]. These and other as yet unforeseen possibilities are likely to be generated from the research focus on mitochondria. The following are a few examples of the emerging directions in mitoepigenetic research.

Methodological issues

As with other fields of epigenetics, advancements in methodologies and assays will remain the driving force for propelling the field of mitoepigenetics. For example, there is still a lingering question as to whether mtDNA methylation, particularly CpG methylation, is a real phenomenon or an artifact [38]. A project, recently funded by the National Human Genome Research Institute [74], will apply a new method of isolating and sequencing human mtDNA and mtDNA-methylation to profile DNA methylation across the mtDNA in an unbiased manner. As discussed earlier, it is preferable and sometimes imperative [20] that mitochondria are isolated and mtDNA separated from nDNA prior to epigenetic characterization. Although nuclear copies of mtDNA (mitochondrial pseudogenes) are more common in plants than in animals [75], they are present in the mammalian genome and steps should be included to avoid their contribution when characterizing mtDNA epigenetic marks. Purifying mtDNA may present a problem in studying mitochondrial epigenetics in the brain, particularly in postmortem human brain samples. When using peripheral samples such as blood cells, one might take advantage of selecting cells that normally do not contain a nucleus but do contain mitochondria and mtDNA (e.g., platelets). Additional considerations should include heteroplasmy of mtDNA—the presence of more than one type of mitochondrial genome (e.g., with and without mutations) within a cell or an

organism. In this context, it has been suggested that in mtDNA methylation events only occur in a certain subset of the multiple copies of mitochondrial genomes within cells, which makes it difficult to consistently detect methylation without high-throughput approaches [76]. Furthermore, heteroplasmy could influence the susceptibility of certain mtDNA variants to epigenetic modifications.

Mechanistic issues

The exact mechanisms of mtDNA methylation and hydroxymethylation are currently unknown. The findings of mitochondrial presence of enzymes involved in the formation of 5mC (mtDNMT1, DNMT3A, DNMT3B) and 5hmC (TET1, TET2), as well as the demonstration of DNMT and TET activities in mitochondria, strongly suggest that similar enzymatic mechanisms of DNA methylation and hydroxymethylation exist in the nucleus and mitochondria. However, several experimental findings indicate that alternative mechanisms might be at play. For example, Bellizzi et al. [34] noted that in cells lacking DNMT1, DNMT3A, and DNMT3B the methylation pattern of mtDNA D-loop did not differ (although methylation was reduced) compared with wild-type cells. These authors observed a dense methylation of symmetrical (CpG) and non-symmetrical (non-CpG) sites within the mitochondrial D-loop, which resembles typical features of RNA-directed DNA methylation. Because this type of DNA methylation has so far been found only in plants and fungi, the authors proposed that the methylation of mtDNA is similar to the epigenetic patterns occurring in plants and fungi rather than those characterizing eukaryotic organisms. Moreover, the findings of TET1 protein in mitochondria does not necessarily imply its major role in 5hmC formation, as evidenced in experiments in which TET1 knockout did not alter the mtDNA 5hmC content [51]. Furthermore, the functional consequences of epigenetic mtDNA markings (e.g., for mtDNA expression and mitochondrial functioning) require further elucidation. To this end, it has been suggested that D-loop methylation regulates the expression of ND1 and ND6 [29] and possibly modulates mtDNA replication [34]. Clearly, further studies are needed to fully characterize the mechanisms operative in mitochondrial epigenetics.

Mitoepigenetic biomarkers

A possible role for epigenetic mtDNA modifications as a biological marker has recently been proposed [1,2,57]. For neuropsychiatric disorders, the usefulness of biomarkers is limited by difficulties in obtaining CNS tissue samples from patients (typically during surgical procedures). Furthermore, mitoepigenetic neuropsychiatric biomarkers have the same advantages and disadvantages as peripheral neuropsychiatric biomarkers in general; a question remains as to the extent to which peripheral biomarkers reflect molecular events (e.g., epigenetics) occurring within the CNS [77]. It was proposed that blood chromatin could serve as a biosensor of the epigenetic milieu and a tool for studies in living psychiatric patients [78]. If this is confirmed, mitochondrial epigenetic characterization could be included in these studies, too.

Conclusion

In spite of its relatively short history, the emphasis on epigenetic components of neuropsychiatric illnesses has already generated an important body of knowledge and is beginning to produce the first

translational results. Compared with the study of neuroepigenetics, research in mitoepigenetics is still in a nascent stage. Nevertheless, several key observations important for the development of this field have been made in the area of neuroscience. Some aspects of mitoepigenetics, such as *mitochondrial epigenetics*, still require full characterization of the mechanisms involved and a conclusive evaluation of functional implications of epigenetic mtDNA modifications. Other aspects, such as mitochondrial regulation of nuclear epigenetics and the epigenetic regulation of nDNA-encoded mitochondrial genes appear to be more advanced.

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Glossary

Heteroplasmy The presence of a mixture of more than one type of mtDNA within a cell. Mutations usually affect some but not all mtDNA molecules within a cell; ultimately, the proportion of normal and mutated mtDNA in different tissues determines the clinical pathology. It is possible that heteroplasmy exists with respect to epigenetic modifications of mtDNA.

Mitochondrion Eukaryotic organelle with its own mtDNA. Mitochondria are primarily involved in energy (e.g., ATP) production but are also critical for a number of other physiological processes. The word "mitochondrion" originates from the Greek *mitos* ("thread-like") and *khondrion*, diminutive of *khondros* ("granule/grain").

mtDNA Mitochondrial DNA, which in many animal species is small (16,295–16,826 bp), circular, intron-free, double-stranded DNA that contains only 37 genes, of which 13 encode polypeptides/proteins. In contrast to nDNA, all cellular mtDNA is exclusively maternally inherited. Each mitochondrion typically contains multiple mtDNA copies (up to 10) and each cell contains multiple mitochondria (up to thousands).

mtDNA haplotypes In the course of human migrations, people have accumulated distinctive variations from the mtDNA of the ancestral "mitochondrial Eve," resulting in haplotypes characteristic of different ethnic groups. It appears that these haplotypes can modulate global DNA methylation and might be associated with susceptibility factors in psychiatric disorders. It is possible that various haplotypes may differentially influence the susceptibility of mtDNA for epigenetic modifications.

Neuropsychiatric disorder A disorder due to dysfunction of the brain spanning the medical specialties of neurology as well as psychiatry.

CHAPTER

Epigenetic Effects of Currently Used Psychotropic Drugs

23

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KEY CONCEPT

- Many currently used psychotropic drugs alter the epigenetic regulation of gene expression in addition to their well-established and frequently discussed mechanisms of action.

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ABBREVIATIONS

BDNF Brain-derived neurotrophic factor
CAPN6 Calpain 6, dipeptidyl-peptidase 10
CHRNA2 Cholinergic receptor, nicotinic, alpha 2
CREB cAMP response element binding protein
DLPFC Dorsolateral prefrontal cortex
DNMT1 DNA methyltransferase 1
ERRg Estrogen-related receptor gamma
FAM126A Member A of family with sequence similarity 126
FGF Fibroblast growth factor
GAD Glutamic acid decarboxylase
GLT1 Glutamate transporter 1
GRM7 Metabotropic glutamate receptor 7
GSK3 Glycogen synthase kinase 3
H3K9 Histone H3 lysine 9
HAT Histone acetyltransferase
HDAC Histone deacetylase
IGF2 Insulin-like growth factor 2
LSD1 Lysine-specific demethylase 1
MAO Monoamine oxidase
MBD Methyl-CpG-binding domain
MeCP2 Methyl-CpG-binding protein 2
miRNA microRNA
NMDA *N*-methyl-D-aspartate
SSRI Selective serotonin reuptake inhibitor
TET Ten–eleven translocation
TH Tyrosine hydroxylase
THRb Thyroid hormone receptor beta
TNFRSF21 Tumor necrosis factor receptor superfamily member 21
VPA Valproic acid

Epigenetic effects of valproic acid

The drug valproic acid (VPA), or its salt sodium valproate, is commonly used in the treatment of epilepsy and bipolar disorder. The effects of VPA on neuronal processes in the brain have been only partly illuminated. The traditional point of view for its mechanism of action is that it inhibits neuronal conduction in the brain through blockage of sodium channels and increase of gamma-aminobutyric acid (GABA). The therapeutic actions of VPA may also be mediated through regulation of tyrosine hydroxylase (TH) expression, as VPA increases TH mRNA and protein levels [1]. TH is involved in the biosynthesis of the catecholamines dopamine, norepinephrine, and epinephrine, and these neurotransmitters are thought to be involved in psychiatric disorders. When VPA is administered to rat cortical neurons, mRNA levels and protein levels of exon 1–9-containing BDNF are elevated, and in astrocytes glial-derived neurotrophic factor is increased [2–4]. In a study in rat glioma cells, VPA failed to induce any change in levels of BDNF mRNA *per se*, but it enhanced the stimulatory effect of serotonin on brain-derived neurotrophic factor (BDNF) gene expression [5]. VPA also affects expression of glutamic acid decarboxylases (GADs), which are responsible for GABA synthesis. Fukuchi et al. [2] found VPA to dose-dependently decrease expression of GAD65, GAD67, and the major $GABA_A$ receptor subunit $\gamma 2$ in rat cortical neurons. However, increases in GAD in response to VPA are also reported [6]. It is likely that these changes are facilitated by epigenetic mechanisms. VPA is a potent histone deacetylase (HDAC) inhibitor of class I and II HDACs, and it increases levels of acetylated histone H3 and H4, increasing gene expression [7]. For these reasons, VPA is one of the most extensively investigated compounds in epigenetic psychopharmacology.

In addition to this straightforward regulatory effect there are several lines of evidence that secondary transcriptional mechanisms play a role. Mounting evidence suggests that by recruiting methylation enzymes VPA also exerts an influence on DNA methylation. Another secondary effect by which VPA influences gene expression is via the transcription factor fosB. VPA-heightened levels of histone H4 acetylation in the striatum increasing transcription of the fosB promoter gene. Striatal

fosB protein levels were also elevated, and because fosB is an ubiquitous transcription factor this likely indicates that VPA enhances genetic expression in the striatum via increased expression of transcription factors such as fosB [8]. Another secondary mechanism for influencing gene transcription is via upregulation of glycogen synthase kinase 3 (GSK3) (similar to lithium), a mechanism of action that is best studied for upregulation of the fibroblast growth factor (FGF) gene promoter [9]. Finally, VPA has been found to selectively modulate microRNAs (miRNAs) [10], which in turn regulate bipolar disorder genes (see later discussion on lithium and microRNAs).

Valproic acid and chromatin marks

A well-balanced regulation of HDACs and histone acetyltransferases (HATs) is essential to well-functioning gene transcription. Alterations in histone acetylation are implicated in many cancers as well as neurodegenerative disorders. HDAC inhibitors differ in their specificity for the separate classes of HDACs, although most target class I and class II HDACs. HDAC inhibitors can be classified into categories based on their chemical structure, and the most important are the hydroxamates, the short fatty-chain acids, and the benzamides. Many HDAC inhibitors exist that have been primarily investigated in the treatment of cancer, such as SAHA, trapoxin, and LBH589 [11]. Unfortunately, the HDAC properties of psychotropic drugs are largely unknown, with the exception of valproate and imipramine.

In the lymphocytes of healthy humans, VPA increased GAD67 mRNA almost fourfold and nearly doubled acetylation of histone H3K9 and H3K14. The effects of VPA were also examined in lymphocytes of bipolar and schizophrenia patients who had received VPA for 4 weeks. VPA significantly increased GAD67 mRNA expression and H3K9, H3K14, and H4 acetylation levels [12,13]. Yu and colleagues [14] found that VPA increased neuronal differentiation both *in vitro* and *in vivo*, together with increased levels of acetylated H3 and H4. However, in contrast to previous results, proliferation of hippocampal progenitor cells was decreased. In neural progenitor cells, VPA increased acetylation of H3 and H4 and induced expression of reelin [6,15,16]. More evidence for H3 acetylation comes from a mouse study that also found a similar effect for the mood stabilizer and antiepileptic lamotrigine for the first time [17].

In an extensive study, Perisic and coworkers [18] examined epigenetic effects of a variety of psychotropic compounds in hippocampal and cortical astrocytes, one of which was VPA. This was the only drug that exerted effects on histone acetylation, inducing dose-dependent global hyperacetylation of histones H3 and H4 and decreasing levels of repressive H3K9 dimethylation. Furthermore, the increased acetylation by VPA caused a fourfold increase in glutamate transporter 1 (GLT1) mRNA and reduced DNA methylation of the GLT1 gene, which concurs with the hypothesis of glutamate hypofunction in bipolar disorder. In addition, VPA induced global demethylation of CpG sites in astrocytes of the cortex and hippocampus (see also below). The effects on acetylation were transient, as they peaked during the first hour of treatment and then declined. Forty-eight hours after VPA treatment acetylation levels had returned to baseline, suggesting that the effects of VPA are fully reversible [18]. Furthermore, VPA increases melatonin MT1 receptor expression in glioma cells. Altered function of melatonin has been implicated in the etiology of depression, providing a possible mechanism of action of VPA in bipolar disorder. In the same study, VPA increased expression of HDAC1, 2, and 3 and of MeCP2, indicating that VPA affects both histone acetylation and DNA methylation [19], and that the upregulation of HDACs and MeCP2 may constitute a compensatory mechanism to VPA-induced hyperacetylation. These studies hint of a property of VPA and other HDAC inhibitors, in general, whereby as a secondary effect DNA methylation is influenced.

Valproic acid and DNA methylation

The effects of VPA on histone acetylation are straightforward, but compelling evidence suggests that there are secondary effects of these altered chromatin marks that influence DNA methylation. DNA methylation was shown to be essential for treatment response to VPA in neuroblastoma [20]. Another study showed the relationship between VPA and methylation of mitochondrial DNA [21]. The authors argued that the potential link of DNA methylation and VPA is via alterations of ten–eleven translocation (TET) enzymes involved in the DNA methylation machinery. A similar mechanism may explain the beneficial effects of VPA to counteract increased methylation of *RELN* and *GAD1* promoters as found in prenatal stressed mice by inhibiting DNMT1 and MeCP2, which are important in the methylation pathways [22]. Such mechanisms may also explain altered DNA methylation in the offspring of mothers that received VPA during pregnancy [23]. Indeed, human studies also suggest that VPA alters DNA methylation enzymes. In the frontal cortex of schizophrenia and bipolar disorder patients with psychosis, DNMT1 mRNA and protein expression preferentially increased in layer I, II, and IV interneurons. Interestingly, DNMT1 expression only increased when patients received antipsychotic monotherapy, and not when they were treated with a combination of VPA and antipsychotic treatment. This strongly suggests that the mechanism of action of VPA is at least partly via DNA methylation [24,25].

Increased expression of *RELN* (a gene important for neurodevelopment) in neural progenitor cells by VPA is associated with increased acetylation of H3 and H4 (also by demethylation of the *RELN* promoter area), as treatment with VPA has been shown to stimulate promoter demethylation in the *RELN* and *GAD1* genes in the cortex and hippocampus of mice [16,26]. Additionally, DNMT1 is downregulated in response to VPA in frontal GABAergic interneurons, likely mediating increased gene expression [6,26]. When VPA is administered together with methionine, it blocks the hypermethylation that is normally induced by methionine alone [27,28]. In addition, VPA attenuates behavioral schizophrenia-like characteristics that are induced by methionine. It is suggested that this is mediated by VPA-induced hyperacetylation of histone H3 at the *RELN* and *GAD1* promoters, which facilitates a state of euchromatin and enhanced DNA demethylase activity. In mice that exhibit methionine-induced hypermethylation, VPA induces demethylation of the *RELN* promoter in the frontal cortex and increased acetylation of H3K9 and H3K14 associated with the promoter. When VPA was administered in conjunction with clozapine or sulpiride, both demethylation and acetylation were further enhanced [29].

Epigenetic effects of antidepressants

Although traditionally the functions of antidepressant drugs have been understood from the perspective of regulation of neurotransmission by covalent binding to receptors, recent studies suggest that the actual regulating function may be facilitated via epigenetic processes for a number of antidepressant drugs (Figure 23.1). Potentially, epigenetic mechanisms could provide an explanation as to why antidepressants are generally only effective after several weeks of treatment. This has never been fully understood, as neurotransmitter levels increase immediately after use. Upon binding to receptors, intracellular cascades may be set into action that ultimately intrude into the cell nucleus and regulate the pattern of histone modifications and DNA methylation. In a recent study, many compounds were

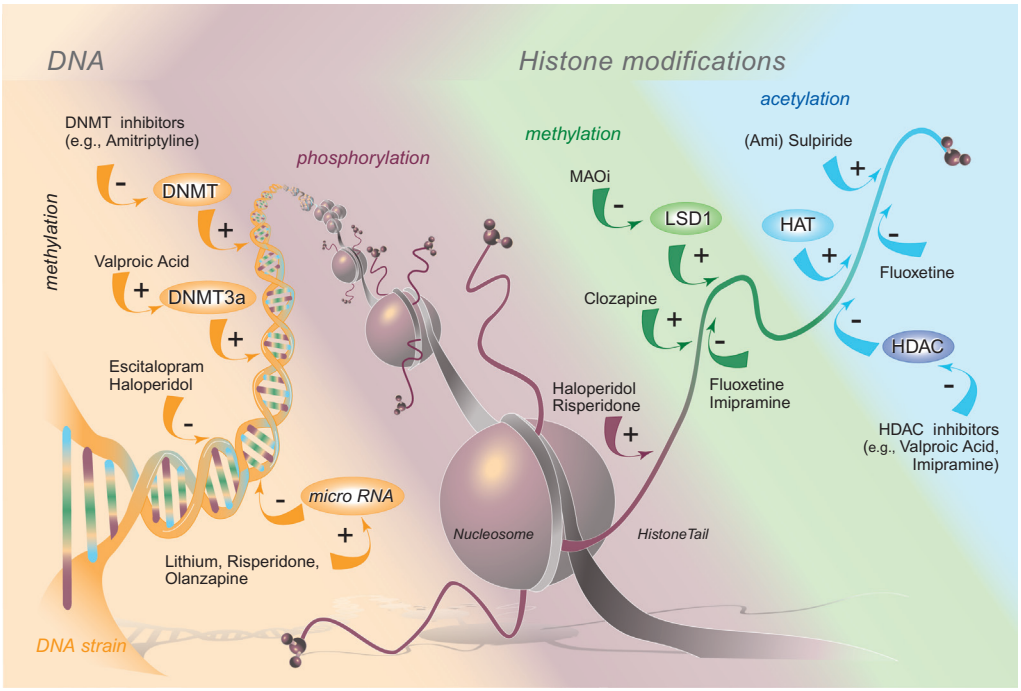

FIGURE 23.1

Schematic diagram showing epigenetic effects of some currently used psychotropic drugs.

tested simultaneously in mice and the effects recorded in different brain regions [17]. The authors showed that the changes of monoamine content accompanied by altered histone deacetylase were restricted to the mood stabilizers VPA and lamotrigine, but increased expression of HDAC2, HDAC3, or HDAC5 was found in a brain region-specific manner. These findings support the possibility that antidepressant-like effects involve changes in HDAC expression.

As early environmental exposures are of particular interest in epigenetic studies, prenatal effects of antidepressants are important. A longitudinal study of 201 babies showed genome-wide differential methylation at a locus in the tumor necrosis factor receptor superfamily member 21 (TNFRSF21) and cholinergic receptor, nicotinic, alpha 2 (CHRNA2) genes associated with antidepressant treatment but not with depressive disorder of the mother, although the differences in methylation levels were small [30].

Imipramine

Imipramine is a tricyclic antidepressant that has been used since the 1950s. Its primary mechanism of action is to inhibit the reuptake of serotonin and norepinephrine, thus elevating the levels of these neurotransmitters in the brain. Recently, epigenetic effects of imipramine have been noted, providing

a deeper understanding into the therapeutic effect of this drug. Because dysfunction of BDNF is often implicated in the etiology of depression, Tsankova et al. [31] investigated the effectiveness of imipramine on epigenetic regulation of the *BDNF* gene in the hippocampi of mice. Chronic social defeat led to repression of histone dimethylation of H3K27 at the *BDNF* exon 3 and 4 promoters, continuing for a month after social defeat. Chronic treatment with imipramine could not reverse this, even though the depression-like behaviors disappeared. However, chronic imipramine did lead to hyperacetylation of H3 at the *BDNF* promoters, mediated by downregulation of *HDAC5*. Moreover, the efficacy of imipramine was blocked by overexpression of *HDAC5*, suggesting that downregulation of *HDAC5* may be essential to the efficacy of imipramine. The authors suggested that, because histone H3 hypermethylation was not affected by imipramine, this remains a possible target for antidepressant therapy. Imipramine also influences epigenetic regulation in the nucleus accumbens by largely reversing repression of dimethylation of H3K9 and H3K27 in the nucleus accumbens and increased phospho–CREB binding induced by social defeat stress. The global pattern of H3 dimethylation in the nucleus accumbens of mice that did not develop depression-like behaviors strongly resembled the dimethylation profile of mice that received chronic imipramine treatment after social defeat. This may indicate that resilient animals somehow naturally overcome the effects of stress on gene expression in the accumbens. Of course, the genes that show dimethylation in resilient mice, but not in mice treated with imipramine, could also be investigated as novel targets for antidepressant medication [32].

Amitriptyline

Amitriptyline is a tricyclic antidepressant. Perisic et al. [18] used this drug in an epigenetic study on rat astrocytes. Amitriptyline induced global DNA hypomethylation without affecting histone acetylation, and this was partially reversible after withdrawal. In addition, amitriptyline strongly reduced the enzymatic activity of DNMT1 without altering DNMT1 protein levels [18], and also inhibited HDAC activity [33].

Clomipramine

Clomipramine is a frequently used tricyclic antidepressant with effects on serotonin and norepinephrine. In mouse nucleus accumbens, it has been shown to lead to an increase in H3 acetylation [17].

Duloxetine

Duloxetine is a serotonin–norepinephrine reuptake inhibitor. It is effective for major depressive disorder and generalized anxiety disorder, and was found to induce significant increases in H3 acetylation in the nucleus accumbens of mice [17].

Fluoxetine

Fluoxetine is a widely used antidepressant, belonging to the class of selective serotonin reuptake inhibitor (SSRIs). In addition, fluoxetine was recently investigated for its epigenetic effects in the hippocampus. Restraint stress was found to induce trimethylation of histone H3K9 and H3K4 in the dentate gyrus in the hippocampus. Treatment with fluoxetine reversed H3K9 but not H3K4 trimethylation,

selectively enhancing gene expression, as only H3K9 trimethylation is a repressor of gene expression [34]. However, postnatal fluoxetine exposure did not change the acetylation or trimethylation of histone H3 at the *BDNF* promoters [35]. Chronic fluoxetine administration to healthy rats decreases acetylation of H3 in three serotonin projection areas: the caudate putamen, the frontal cortex, and the dentate gyrus of the hippocampus. In addition, expression of the methyl-binding proteins MeCP2 and MBD1 was increased, accompanied by increased HDAC2 expression, further inhibiting transcriptional activity in these brain regions [36]. In mice that have been subjected to social defeat stress, a global characteristic pattern of gene transcription is induced in the nucleus accumbens. Treatment with fluoxetine reverses this in the same way as HDAC inhibitors, suggesting a similar mechanism of action [37].

Escitalopram

The SSRI escitalopram was able to reverse hypermethylation at the P11 (S100A10) locus in a genetic rodent model of depression. Expression of P11 is associated with depression, suggesting a putative epigenetic mechanism of escitalopram efficacy [38].

Monoamine Oxidase Inhibitors

The monoamine oxidase (MAO) enzymes catalyze the breakdown of the monoaminergic neurotransmitters serotonin, dopamine, epinephrine, and norepinephrine. Methylation status of the MAO A promoter (detected in white blood cells) can reliably predict MAO function in the brain. MAO inhibitors form a group of effective antidepressant medications. They reduce the activity of MAO and this increases availability of neurotransmitters. MAO inhibitors are capable of enhancing histone methylation by breaking down lysine-specific demethylase 1 (LSD1), a histone demethylase that is structurally similar to MAO A and B [39]. LSD1 specifically demethylates mono- and dimethylated H3K4 and H3K9, which are inhibitory modifications [40]. The MAO inhibitors phenelzine and tranylcypromine both inhibit demethylation of histone H3K4, resulting in a global increase in H3K4 methylation. Interestingly, tranylcypromine was 10 times more effective in inhibiting LSD1 than MAO A or B, suggesting that selective LSD1 inhibitors might be effective in treating depression. Other experimental MAO inhibitors also increase H3K4 methylation, but the effects of such drugs on H3K9 methylation have not been investigated to date [41].

Epigenetic effects of antipsychotics

There is a considerable amount of interest in the epigenetic effects of antipsychotic drugs and the first evidence for epigenetic involvement dates back to 1976 when Stefanis et al. [42] showed that pimozide normalized the chromatin staining patterns in nuclei of white blood cells of schizophrenia patients. There is likely to be a link between antipsychotic use and HDAC activity based on the reported efficacy of the combination of HDAC inhibitors with antipsychotics, particularly clozapine and sulpiride [24]. Among the potential HDACs that may be affected by antipsychotic drugs, HDAC2 is the most promising candidate based on studies in mice and postmortem brain of schizophrenia patients. Treatment with chronic clozapine or risperidone, but not haloperidol, was associated with

downregulation of the metabotropic glutamate 2 receptor and decreased histone acetylation at its promoter in mouse and human frontal cortex, similar to the effect of upregulating HDAC2. In another study, olanzapine was shown to upregulate HDAC2 and HDAC3 in the striatum, significantly increasing HDAC3 in the cingulate cortex and significantly increasing HDAC5 in the amygdala [17]. In contrast, other studies have shown that antipsychotic drug treatment, including the atypical antipsychotic clozapine, did not affect HDAC1 levels in prefrontal cortex of mice [43]. The most recent perspective is the involvement of miRNAs in the efficacy and adverse effects of antipsychotics [44].

Haloperidol

Haloperidol is a widely used typical antipsychotic that preferentially antagonizes dopamine D2 receptor activity. In addition, some evidence indicates that haloperidol induces histone modifications to achieve its therapeutic effects. In mice, histone phosphoacetylation is observed in the striatum after striatal infusion of haloperidol. Specifically, histone H3 at serine 10 is phosphorylated and H3K14 is acetylated, and this is mediated by the NMDA receptor, as administration of a NMDA receptor antagonist blocks phosphoacetylation. The same results were seen following administration of the D2 receptor antagonist raclopride [45]. Another recent study also observed increased H3 phosphorylation in the striatum, but no changes in acetylation were found. Two more studies also reported an increase in mouse striatal H3 phosphorylation in response to haloperidol, although acetylation levels remained unchanged [46,47]. Furthermore, one study reported that haloperidol decreased global DNA methylation levels in the brain of female rats [48]. However, haloperidol did not influence methylation of the *RELN* and *GAD1* gene promoters in the frontal cortex or striatum [29].

Pimozide

In one of the first epigenetic studies in psychopharmacology it was shown that pimozide normalized chromatin-staining patterns in nuclei of white blood cells of patients with schizophrenia [42].

Clozapine

Treatment with the atypical antipsychotic clozapine, which blocks dopamine D1, D2, and 5HT2 receptors, increases GAD67-associated trimethylation of H3K4 threefold in the rat cerebral cortex. In addition, mRNA expression of the H3K4-specific histone methyltransferase gene Mll1 was increased, and Mll1 occupancy at the *GAD1* promoter had doubled. In the prefrontal cortex of humans who had been treated with clozapine, H3K4 trimethylation at the *GAD1* gene had increased twofold. GAD is an enzyme that catalyzes the decarboxylation of glutamate to GABA and CO_2. As the epigenetic modifications at the GAD64 locus is associated with transcriptional activation, these findings indicate that the therapeutic action of clozapine relies at least in part on increased GABAergic activity mediated by histone methylation [49]. Further support for this hypothesis comes from the finding that methionine-induced *RELN* promoter hypermethylation was strongly decreased after administration of clozapine, together with an increase in promoter-associated H3K9 and H3K14 acetylation [29]. Clozapine was also able to reverse the effects of the mGlu2/3 receptor agonist LY379268 on Gadd45-β hypermethylation [50]. Clozapine further increased H3 acetylation in the nucleus accumbens of mice [17] and may influence the HDAC enzymes directly.

Olanzapine

Olanzapine increased H3 acetylation in the nucleus accumbens in mice and caused an increase in HDAC2 and HDAC3 in the striatum and a significant increase in HDAC3 in the cingulate cortex. It also caused a significant increase in HDAC5 in the amygdala, testifying to brain-region-specific responses [17].

Sulpiride and amisulpiride

Two structurally similar antipsychotics, sulpiride and amisulpiride, act by antagonizing the dopamine D2 receptor. In addition, they are highly effective in increasing acetylation of H3 associated with *RELN* and *GAD1* gene promoters in the frontal cortices of mice. These drugs have the same effect in the hippocampus and the striatum, but much higher doses are needed, indicating there is differential sensitivity to these drugs across different regions of the brain [51]. In the frontal cortex and striatum of mice that displayed methionine-induced hypermethylation of the *RELN* promoter, methylation was strongly decreased after administration of sulpiride, together with an increase in promoter-associated H3K9 and H3K14 acetylation [29].

Risperidone

Risperidone is another atypical antipsychotic that antagonizes D2 receptors but has greater affinity for 5HT2 receptors. It induces global phosphoacetylation of H3 in the striatum, mediated by the NMDA receptor, showing that dopamine, serotonin, and glutamate act in synchrony to influence chromatin regulation [52].

Antipsychotics and microRNAs

A new perspective on epigenetic mechanisms involved in antipsychotic treatment is studies where the association between miRNA changes and drug efficacy and side effects are investigated, focusing on the recently identified miRNAs that are associated with schizophrenia [53–55]. In a comprehensive study, Santarelli and coworkers reported downregulation of miRNA in mouse brains after haloperidol and olanzapine treatment. There results suggest that expression levels of miRNAs associated with schizophrenia may be normalized by antipsychotic drugs. For example, miR-193, miR-223, and miR-544, all previously observed to be elevated in the DLPFC in schizophrenia, are downregulated by olanzapine treatment, suggesting that olanzapine may help reverse dysregulation of these miRNAs [44]. On a similar note, but in human plasma samples, the expression levels of miR-365 and miR-520c-3p were significantly downregulated after 1 year of risperidone treatment. However, no significant correlations between the clinical symptoms and the expression levels of these two miRNAs were reported [56].

Epigenetic effects of Lithium salts

Lithium has been the treatment of choice for bipolar disorder since the 1970s. Its molecular mechanisms of action are not completely understood, although it has been clear for some time that it

inhibits glycogen synthase kinase 3 (GSK3). This enzyme decreases protein activity by phosphorylation and promotes cellular proliferation in tumors; hence, lithium is being investigated for applications in cancer therapy [57]. Recently, the neuroprotective properties of lithium have led to a range of possible applications of lithium in addition to bipolar disorder [58]. In the context of epigenetics it is important to note that GSK3 is a strong modifier of the expression of the *de novo* DNA methyltransferase DNMT3a2, causing mis-expression of the imprinted genes *IGF2, H19*, and *IGF2* and hypomethylation of their corresponding imprinted control regions [59,60]. Inhibition of GSK3 by lithium or other compounds results in reduced DNA methylation at these imprinted loci. In rat cortical neurons, lithium enhances the expression of BDNF promoter IV by inhibiting GSK3 [61]. Combined treatment with lithium and VPA potentiated serine phosphorylation of GSK3 alpha and beta isoforms and led to enhanced inhibition of GSK3 enzyme activity and consequently to demethylation.

A similar secondary effect of lithium can be seen in relation to histone acetylation. When injected into the central nucleus of the amygdala, expression of the transcription factor c-*fos* is increased, and this is accompanied by highly increased acetylation and phosphoacetylation of histone H3K14, with a time course slightly preceding and corresponding to c-*fos* induction. Acetylation and phosphoacetylation also colocalized with c-*fos* expression in the central amygdala. However, no changes in acetylation were induced in other nuclei of the amygdala, indicating that lithium acts region specifically [62]. In mice, lithium increases H3 acetylation in the accumbens [17].

Interestingly, lithium has also been linked with altered miRNA expression. In the rat hippocampus, chronic treatment with either lithium or VPA altered expression of several but not all miRNAs [10]. Some of the unregulated miRNAs (miR-24, -34a, -128) target genes that have previously been implicated in bipolar disorder: calpain 6, dipeptidyl-peptidase 10 (*CAPN6)*, estrogen-related receptor gamma (*ERRg*), member A of family with sequence similarity 126 (*FAM126A*), metabotropic glutamate receptor 7 (*GRM7*), and thyroid hormone receptor beta (*THRb*). These relationships were confirmed in human lymphoblast cell lines and mice. As further evidence of the relevance of miRNAs for treatment with lithium, Rong et al. [63] showed that effective treatment of mania was accompanied with a normalization of miRNA-134 in the plasma of patients. Another miRNA, let-7b, was altered by lithium treatment. Interestingly, this miRNA is involved in regulation of neurodegeneration via Toll-like receptors [64] and in bipolar disorder via muscarinic M_1 receptors [65].

Conclusion

Many commonly used psychotropic drugs exert an influence on the epigenome and more compounds are added every few months. Table 23.1 summarizes the most recent state of knowledge. Although for a fair amount of these influenced epigenetic mechanisms the molecular targets have been identified, the studies are generally limited to candidate pathways for which previous evidence exists. As a result we remain unaware of the full extent of the effects of these epigenetic alterations, and epigenetic pharmacological studies therefore have only started to scratch the surface of what is potentially a huge field. That said, we also currently do not know how the modified transcription relates to epigenetic alterations translate to clinical practice. Particularly in human studies, we do not know whether the efficacy of drugs is mediated by the epigenetic and transcriptional changes that we find in laboratory studies. It is in these limitations of our knowledge that the challenge emerges to systematically study: (1) the epigenetic effects of compounds in a broad perspective, from miRNAs to DNA

Table 23.1 Epigenetic Effects of Currently Used Psychotropic Drugs and Investigational Epigenetic Drugs

Drug	Histone Acetylation	Histone Monomethylation	Histone Dimethylation	Histone Trimethylation	Histone Phosphorylation	Histone Phosphoacetylation	microRNA	DNA Methylation
Antidepressants								
Imipramine	X [31]		X [32]					
Amitriptyline								X [18]
Fluoxetine	X [36]			X [34]				
Escitalopram								X [38]
Clomipramine	X [17]							
Duloxetine	X [17]							
MAO inhibitors		X [41]	X [41]					
Antipsychotics								
Haloperidol					X [46,47]	X [45]		X [48]
Clozapine	X [29]			X [49]				X [29]
Olanzapine	X [17]						X [44]	
(Ami)sulpiride	X [45,51]							X [29]
Pimozide	X [42]							
Risperidone						X [45]	X [56]	
Valproic acid	X [8,12, 13,15,16, 18,26–28,49]		X [18]					X [8,16, 18,24, 26,27,29]
Lithium	X [17]					X [62]	X [73]	

Note: The numbers in parentheses refer to references for those particular drug studies. See also references [3,6,7,62, and 66–72] for studies based on investigational epigenetic drugs.

methylation and chromatin marks; (2) the molecular relevance of these effects, not limited to a single pathway; and (3) the clinical relevance of these changes. It is clear that considering the scale of such investigations, they entail a tremendous challenge. However, in light of the vast knowledge that has already been obtained for each of these steps, it is also clear that many of the pieces of the puzzle are already available and that integration of existing knowledge is an important requirement for progress.

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Glossary

Antidepressant A drug used to treat major depressive disorder.
Antipsychotic A drug used to treat psychotic disorders such as schizophrenia.
Monoamine oxidase An enzyme that catalyzes the metabolism of monoamines.
Psychopharmacology The study of drugs used to treat psychiatric disorders.
Psychotropic drug A drug used to treat psychiatric disorders.

CHAPTER

DNA Methyltransferase Inhibitors and Psychiatric Disorders

24

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KEY CONCEPTS

- Aberrant DNA methylation is thought to underlie several psychiatric disorders, including schizophrenia, depression, bipolar disorder, and postraumatic stress disorder. Drugs that inhibit the enzymes DNA methyltransferases (DNMT inhibitors) may be useful for treating these disorders.
- The currently available DNMT inhibitors lack specificity. To improve specificity and toxicity profiles of DNMT inhibitors it will be important to develop drugs that target specific DNA methyltransferases. Such drugs should also be able to adequately cross the blood–brain barrier.
- A better understanding of how epigenetic mechanisms contribute to specific psychiatric disorders is needed.
- Epigenetic biomarkers that assess epigenetic dysregulation in connection to a specific (endo) phenotype in a particular patient are needed.

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ABBREVIATIONS

BDNF Brain-derived neurotrophic factor
COMT Catechol-*O*-methyltransferase
CpG Cytosine–phosphate–guanine
DNMT DNA methyltransferase
GABA gamma-aminobutyric acid
GAD67 Glutamatic acid decarboxylase 67
HDAC Histone deacetylase
HTR2A Serotonin receptor type 2A
MeCP2 Methyl-CpG-binding protein 2
NAc Nucleus accumbens
PCNA Proliferating cell nuclear antigen
PHD Plant homeodomain
PP1 Protein phospatase 1 gene
SAM *S*-adenosyl-L-methionine
SOX10 Sex-determining region Y box containing gene 10
SSRI selective serotonin reuptake inhibitor

Introduction

Pharmacological treatment of psychiatric disorders includes a variety of drugs such as antipsychotics, antidepressants, mood stabilizers, and anxiolytics [1]. These drugs mainly target monoaminergic neurotransmitter systems acting via several mechanisms: (1) by altering the activity of monoaminergic receptors; (2) by inhibiting the reuptake of neurotransmitters into a presynaptic terminal; and (3) by inhibiting the enzymes involved in the synthesis and breakdown of monoamine neurotransmitters. Although the current drugs are effective in relieving the symptoms in a large number of psychiatric patients, it usually takes several weeks for them to become fully effective, and still a substantial number of patients are partial responders or even treatment resistant. The development of new psychoactive drugs with similar mechanisms of action did not prove to be a successful strategy for improving the clinical effectiveness; therefore, there is a great need for the introduction of novel compounds with a substantially different mode of action. For this, we must improve our understanding of the etiology and pathophysiology of psychiatric disorders. In recent years, there has been a growing interest in studying the possible involvement of epigenetic mechanisms in the pathogenesis of neuropsychiatric disorders, and epigenetic-based therapeutics have emerged as promising new drugs to treat schizophrenia, depression, and bipolar disorders, among others [2–4].

Epigenetic mechanisms, such as DNA methylation and histone modifications, control the state of chromatin in the vicinity of gene regulatory regions and thereby directly regulate gene expression in the developing and adult brain [5]. Epigenetic marks are dynamic and responsive to environmental cues throughout life, providing a biological substrate through which the environment can shape brain structure and function over the life course [6,7]. Therefore, epigenetic dysregulation may occur in response to adverse environments, but also due to genetic factors or random errors in developmental programming, and can contribute to brain dysfunction. The disruption of epigenetic mechanisms with consequent gene expression changes in various brain regions has been implicated in the pathophysiology of several psychiatric disorders, including schizophrenia, depression, bipolar disorder, and posttraumatic stress disorder [4,8,9]. Epigenetic alterations are attractive therapeutic targets because they are potentially reversible by drugs that target epigenetic machinery and that are able to affect the epigenome. Some epigenetic drugs, such as DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors, are already accessible as they are being used or are in development for many diverse diseases, including certain types of cancer and neurodegenerative disorders [10,11]. This chapter focuses on DNA methylation and DNMTs as possible new targets for the treatment of several psychiatric disorders, including schizophrenia, depression, bipolar disorder, and posttraumatic stress disorder (PTSD).

DNA methylation and DNMTs

DNA methylation is a critical epigenetic mechanism involved in regulation of gene expression [12]. Methylation occurs at position 5 of the cytosine residue (Figure 24.1) and primarily in the context of CpG dinucleotides, although recent evidence suggests that non-CpG methylation is also relevant [13]. DNA methylation is typically associated with transcriptional silencing. DNA methylation either can directly impede the binding of transcription factors or can recruit methyl-CpG-binding proteins and other repressors to gene regulatory regions, resulting in closed chromatin structure that is not permissive to transcription. DNA methylation is catalyzed by the enzymes known as DNA methyltransferases (DNMTs) (Figure 24.1) [12].

The family of mammalian DNMTs includes five members: DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L (Figure 24.2). These proteins have a very similar structure, containing a catalytic C-terminal domain and regulatory N-terminal domain (Figure 24.2) [14]. However, only DNMT1, DNMT3A, and DNMT3B are proven to be catalytically active *in vivo*. DNMT3A and DNMT3B are generally recognized as *de novo* methyltransferases, as they are responsible for introducing cytosine methylation at previously unmethylated CpG sites. In contrast, DNMT1 is generally regarded as the maintenance methyltransferase; it shows a preference for hemimethylated DNA and is responsible for copying DNA methylation patterns from parent to daughter strands during DNA replication [15]. However, increasing evidence suggests that all three enzymes are capable of methylating both unmethylated and hemimethylated DNA substrates [12].

In general, the enzymatic reaction mechanism of DNMTs involves a transfer of a methyl group from the cofactor and methyl donor *S*-adenosyl-L-methionine (SAM) to the 5-position of the cytosine ring (Figure 24.1). The catalytic (methyltransferase) domain shows a high degree of homology among different mammalian DNMTs (Figure 24.2). The highly conserved motifs I, IV, VI, VIII, and X, together with motif IX, appear to be particularly important for the enzymatic function. Motif I contains the binding site for SAM and, together with motifs IV, VI, and X, constitutes the SAM binding pocket. Motif IV likely contains the enzyme active site [14,16]. DNMTs do not exhibit any sequence specificity beyond the CpG dinucleotide [17]. The sequence recognition domain likely resides in the region between motifs VIII and IX that varies in size between DNMTs [14]. The limited sequence specificity of DNMTs suggests that the targeting of these enzymes to specific genomic regions probably occurs through protein–protein interactions [17]. For instance, during DNA replication, DNMT1 is recruited to the replication fork via its interaction with proliferating cell nuclear antigen (PCNA) through the PCNA-binding domain in the N-terminal region (Figure 24.2).

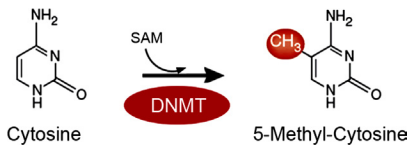


FIGURE 24.1

DNA methylation. Methylation occurs at position 5 of the cytosine base. It is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group from the methyl donor *S*-adenosyl-L-methionine (SAM) to the 5-position of the cytosine ring.

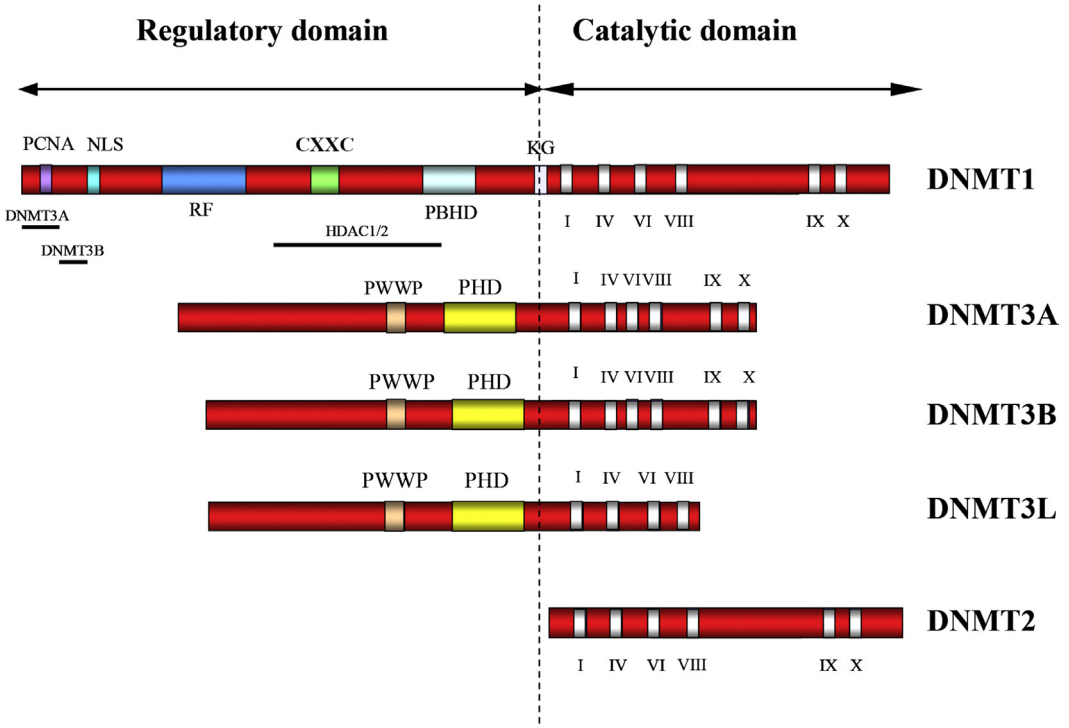


FIGURE 24.2

The mammalian DNA methyltransferase (DNMT) family. The C-terminal domain of DNMTs is the catalytic (methyltransferase) domain that consists of six conserved motifs containing the SAM binding pocket and the enzyme active site (except in the case of DNMT3L). The N-terminal domain is the regulatory domain. The DNMT1 regulatory domain contains the PCNA-binding domain (PCNA), nuclear localization signal (NLS), a region for targeting the replication fork (RF), CXXC-type zinc finger domain (CXXC), and a polybromo-homologous domain (PBHD). The N-terminal domain of DNMT1 can also interact with DNMT3A and DNMT3B as well as with histones deacetylases (HDAC1/2); the sites responsible for these interactions are depicted. DNMT3A and DNMT3B (as well as DNMT2) contain all conserved sequence motifs in the catalytic domain. In addition, all three DNMT3 proteins share the PWWP (proline–tryptophan–tryptophan–proline) domain involved in DNA binding and the PHD (plant homeodomain) domain implicated in transcriptional repression.

Importantly, DNMT functions extend far beyond what was originally thought. In addition to their catalytic function, DNMTs are now known to act as transcriptional repressors independent of their methylation activity [17]. They are directly involved in transcriptional repression via association with the promoter regions and interactions with other proteins. It has been shown that DNMTs can interact with one another, as well as with other repressor proteins, generally through their regulatory N-terminal domains (Figure 24.2). Many of their interacting partners are chromatin-modifying enzymes, including histone deacetylases (HDACs) and histone methyltransferases. For example,

DNMT3 proteins interact with HDACs through the plant homeodomain (PHD) in the N-terminal region (Figure 24.2). The interaction of DNMT proteins with chromatin modifiers implies that DNMT-mediated transcriptional repression involves the modification of chromatin structure [14,17].

DNMT2 and DNMT3L are two additional DNMT-related proteins that have been identified in mammals (Figure 24.2). DNMT2 possesses a conserved DNMT catalytic domain and exhibits weak DNA methyltransferase activity *in vitro*. However, this protein has not been shown to have an effect on DNA methylation *in vivo*, and its real function has yet to be determined [14,18]. DNMT3L has a PHD domain typical of DNMT3 family members, but lacks conserved motifs IX and X in the catalytic domain and thereby does not possess intrinsic methyltransferase activity. However, this protein physically associates with DNMT3A and DNMT3B and enhances their catalytic activities. DNMT3L cannot bind to DNA directly and probably does not act by recruiting *de novo* DNMTs to DNA. However, similar to DNMT3A and DNMT3B, this protein has also been shown to act as a transcriptional corepressor and to form repressive complexes with HDACs dependent on its PHD domain [14].

DNA methylation and DNMTs in the developing and adult brain

DNA methylation and DNMTs are critical for normal mammalian development and cellular differentiation. Knockout of *Dnmt1* or *Dnmt3B* in mice results in embryonic lethality, while *Dnmt3a* knockout mice die around 4 weeks after birth [19,20]. Not surprisingly, DNA methylation is also involved in gene expression programming that is essential for neuronal differentiation and brain development [21–23]. For example, during neurogenesis, neural precursor cells express factors that facilitate transcription of neuronal genes, whereas some glial-specific genes are silenced by DNA methylation [23]. *In vivo* evidence for this was provided by the analysis of a conditional knockout of the *Dnmt1* gene in neural progenitor cells in the developing brain [24]. Brains of these mice exhibited aberrant upregulation of glial-specific genes, reduced numbers of neurons, and precocious astroglial differentiation, implying that the neuro- to gliogenic switch involves DNA demethylation. Further evidence that DNA methylation is important for brain development comes from the studies of Rett syndrome, which is one of the most common forms of mental retardation in females. Approximately 90–95% of cases of this disorder are caused by a mutation in the gene encoding methyl-CpG-binding protein 2 (MeCP2), which is involved in DNA methylation-mediated gene repression [25]. As *MeCP2* mutations result in a primarily neuronal phenotype, this shows that this protein and DNA methylation have particularly important roles in the developing brain.

In agreement with their primary role in development and cellular differentiation, the expression of DNMTs is very high during development and in proliferating cells, and then it normally declines in terminally differentiated cells. However, the brain seems to be an exception; *Dnmt3b* is the only DNMT-related gene that is highly expressed in the developing brain and practically ceases to be expressed by postmitotic neurons [5]. In contrast, *Dnmt1* and *Dnmt3a* are highly expressed by postmitotic neurons in various brain regions [26,27], which implies that DNA methylation has an important role in the adult brain. Indeed, in the last decade, a large body of evidence has shown that DNA methylation plays an active role in dynamic regulation of gene expression in the adult brain [28,29]. Together with histone modifications, DNA methylation participates in neuronal activity- and experience-dependent regulation of genes involved in synaptic plasticity, learning, and adult neurogenesis [28,30]. Accordingly, a specific genetic deletion of *Dnmt1* and *Dnmt3a* in mature forebrain neurons

results in globally altered DNA methylation and gene expression, abnormal hippocampal plasticity, and memory deficits [26]. In addition, an overexpression of *Dnmt3a* levels in the adult nucleus accumbens affects global levels of DNA methylation and spine plasticity in this brain region as well as emotional behavior [31]. However, the large amount of evidence that DNA methylation plays an important role in synaptic plasticity, memory formation, and behavior was provided using the drugs that inhibit DNMTs, as will be explained later.

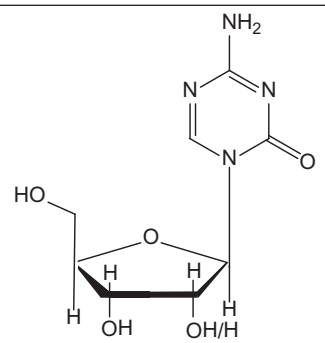
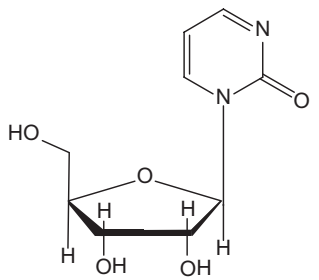
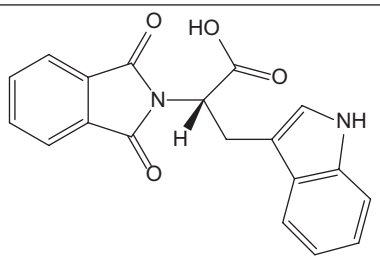
DNMT inhibitors: currently available drugs and their mechanisms of action

DNA methyltransferase (DNMT) inhibitors are drugs that are used to study the role of DNA methylation in different tissues and model systems, and these compounds represent a possible therapeutic option for disorders that involve altered DNA methylation. The most frequently used DNMT inhibitors are presented in Table 24.1. Based on their structure and mechanism of action, they are classified as either nucleoside analog inhibitors or non-nucleoside inhibitors [32].

Nucleoside analog inhibitors closely resemble the structure of cytidine (Table 24.1; also see Figure 24.1). 5-Azacytidine and its deoxy analog, 5-aza-2′-deoxycytidine (decitabine), are analogs of cytidine that have a nitrogen atom in place of carbon in the fifth position of the ring [33]. These drugs have been used for quite a long time and are the first DNMT inhibitors that are FDA-approved for the treatment of myelodysplastic syndromes [34]. Zebularine is a newer and more stable cytidine analog that lacks the amino group at carbon 4 (C-4). All three cytidine analogs function as mechanism-dependent suicide inhibitors of DNMTs [33]. To be effective, they must first be metabolized and phosphorylated to deoxynucleotide triphosphates and then incorporated into replicating DNA in place of cytidine. DNMTs recognize the modified bases as natural substrate and are able to initiate the methylation reaction. However, the analog prevents the resolution of a covalent reaction intermediate and, as a result, the enzyme becomes trapped and degraded. DNA demethylation effect of cytidine analogs is a direct consequence of DNMT depletion; if cells divide and replicate DNA in the presence of diminished DNMT levels, this results in progressive DNA demethylation [35].

However, nucleoside analogs can also induce DNA demethylation in postmitotic neurons, although their mechanism of action in the absence of DNA replication is not clear. There are a few possible explanations for this effect. First, these drugs may reduce DNMT expression in the brain through mechanisms other than covalent trapping and protein degradation during DNA synthesis. As will be described later, it has been reported that zebularine can reduce DNMT expression at the mRNA level [36]. In addition, nucleoside analogs may get incorporated into DNA of postmitotic cells during the DNA repair process, although this is very poorly understood. Finally, studies in cancer cell lines have shown that transcriptional effects of 5-aza-2′-deoxycytidine more closely resemble the effects of HDAC inhibitors (Trichostatin A) than the effects of DNMT-targeted knockouts [37]. This implies that both classes of epigenetic inhibitors may trigger an active DNA demethylation process through an upstream mechanism involved in regulation of both DNA methylation and histone acetylation [32]. Indeed, many studies have shown overlapping effects of DNMT inhibitors and HDAC inhibitors on the epigenome [38–40], although the underlying mechanisms remain unknown. In addition to epigenetic effects, the mechanism of action of cytidine analogs likely involves several additional cellular pathways; therefore, their specificity is relatively low. Consequently, these drugs are

Table 24.1 DNA Methyltransferase Inhibitors

Compound	Proposed Mechanism	Structure
Nucleoside analog inhibitors		
5-Azacytidine 5-Aza-2′-deoxycytidine (decitabine)	Covalent enzyme trapping	
Zebularine	Covalent enzyme trapping	
Non-nucleoside inhibitors		
RG108	Non-covalent, direct enzyme inhibition	

characterized by substantial cellular and clinical toxicity, which has driven the development of novel and more specific drugs, particularly those that may directly target DNMTs [32].

RG108 is a non-nucleoside compound (Table 24.1) that represents the first rationally designed small-molecule inhibitor of DNMTs [41]. This drug acts through a direct inhibitory mechanism and was identified in an *in silico* screen with an active site model of the human DNMT1 enzyme [32]. RG108 was the first compound that showed DNMT inhibitor activity in both a cell-free *in vitro* assay with purified recombinant DNMT and human cancer cell lines, which confirms that it is capable of

direct enzyme inhibition [42]. In addition, it has shown very little toxicity both *in vitro* and *in vivo*. RG108 has also been used in neuroscience research and has been shown to induce DNA demethylation in the brain with consequent changes in gene expression [36,43]. However, RG108 is a highly hydrophobic compound, which is an important limitation to its more widespread application [32]. Indeed, this compound has only been used experimentally via direct infusion into specific brain regions [31,36,43]. There is a great need for additional non-nucleoside, direct DNMT inhibitors, and improved RG108 derivatives are under development [44].

Experimental use of DNMT inhibitors in neuroscience research: relevance for learning and memory

The first use of DNMT inhibitors in neuroscience research was to investigate a possible role of DNMTs and DNA methylation in the adult brain, specifically in the context of learning and memory. Using hippocampal slices of adult mice, Levenson and colleagues [45] demonstrated that the acute administration of the DNMT inhibitor zebularine resulted in a decreased DNA methylation of the reelin and brain-derived neurotrophic factor (*Bdnf*) genes, two genes that are strongly implicated in synaptic plasticity. Zebularine and another DNMT Inhibitor, 5-aza-2′-deoxycytidine, were also able to block hippocampal long-term potentiation, further demonstrating that DNMT may play a role in regulating the induction of synaptic plasticity in the mature brain. This was also the first study to show that cytidine analogs are able to induce DNA demethylation in postmitotic neurons and, together with the previous *in vitro* studies [46], suggested that DNA methylation is dynamically regulated in postmitotic neurons and may be critical for activity-dependent gene regulation and neural plasticity. To further explore this hypothesis, Sweatt and colleagues performed *in vivo* studies by infusing DNMT inhibitors directly into the hippocampus of adult animals [47,48]. Hippocampal administration of DNMT inhibitors blocked memory consolidation after contextual fear conditioning and prevented associated DNA methylation and mRNA expression changes in memory-related genes, including protein phospatase 1 (*PP1*), reelin, and *Bdnf*. A more recent study also showed that intracortical infusion of 5-aza-2′-deoxycytidine, zabularine, or RG108 was able to disrupt DNA methylation-dependent long-term fear memory [43]. Together, these studies were instrumental in establishing the role of DNA methylation in memory formation and maintenance in the adult brain [28]. In addition, they paved the way for the use of DNMT inhibitors in neuroscience and opened the possibility that these drugs may be useful in treating neuropsychiatric disorders associated with aberrant DNA methylation. Downregulation of gene expression caused by DNA hypermethylation represents an attractive therapeutic target, as it can be potentially reversed by the drugs that target DNMTs.

Therapeutic potential of DNMT inhibitors in schizophrenia

Schizophrenia is a complex psychiatric syndrome that involves several groups of symptoms with likely different underlying pathophysiologies [49]. Current antipsychotic drugs primarily target dopamine and serotonin receptors and are efficacious in treating positive symptoms, while negative symptoms and cognitive deficits in schizophrenia are largely unaffected by these drugs [50]. Recently, there has been increasing interest in studying epigenetic mechanisms that may contribute to the development of this disorder with particular emphasis on DNA methylation changes [8,51].

The first evidence that DNA methylation may be involved in the pathophysiology of schizophrenia came from the early clinical studies in the 1960s in which schizophrenic patients were administered methionine, a precursor of the methyl group donor SAM involved in DNA methylation reaction. These studies showed that the administration of methionine, usually in combination with a monoamine oxidase inhibitor, exacerbated psychotic symptoms in nearly 64% (62 of 107) of patients with chronic schizophrenia [52,53]. In agreement with these findings, more recent postmortem human brain studies provided solid evidence that schizophrenia is associated with epigenetic dysregulation [54,55]. DNA methylation changes have been reported in the regulatory regions of several genes of potential relevance to schizophrenia, including reelin (*RELN*) [56,57], catechol-*O*-methyltransferase (*COMT*) [58], serotonin receptor type 2A (*HTR2A*) [59], and sex-determining region Y box containing gene 10 (*SOX10*) [60]. In addition, a genome-wide study by Mill et al. [61] examined frontal cortices of psychotic patients and control subjects, and found differential DNA methylation patterns at numerous loci, including several genes involved in gamma-aminobutyric acid (GABA)ergic and glutamatergic neurotransmission and brain development. In the majority of the studies, DNA methylation changes were inversely correlated with changes in gene expression, strengthening the hypothesis that epigenetic alterations could contribute to brain dysfunction in schizophrenia by changing mRNA and protein abundance in particular neuronal and/or glial cell types.

Among different neuronal populations, deficits in GABAergic neurons have been extensively studied in schizophrenia and have been linked to epigenetic dysfunction [8]. The reduced gene expression of two GABAergic neuronal markers, reelin and glutamic acid decarboxylase 67 (GAD67), has been one of the most consistent findings reported in the postmortem studies of schizophrenia patients' brains [62–65]. Reelin is a critical neurodevelopmental protein involved in neuronal migration and positioning; it continues to be expressed in adult GABAergic neurons with a role in synaptic plasticity and memory formation [66]. GAD67 is one of the two rate-limiting enzymes that catalyzes the synthesis of GABA, and it plays a central role in the regulation of GABA-mediated neuronal signaling. Both reelin and GAD67 colocalize with DNMT1 in GABAergic neurons. Importantly, two studies using different methodologies showed that DNMT1 mRNA levels are upregulated in cortical GABAergic neurons of schizophrenia patients [67,68]. Together, these findings led to a hypothesis that the reduced mRNA levels of reelin and GAD67 (and possibly some other GABAergic genes) may be due to increased promoter methylation mediated by upregulated DNMT activity in schizophrenic brains [8]. This hypothesis is further supported by the finding that the reelin gene is indeed hypermethylated in schizophrenia [57]. Therefore, methionine could worsen the symptoms in schizophrenia patients by providing an increased amount of substrate for DNMT1, further facilitating DNA methylation of critical psychosis-associated genes.

The epigenetic hypothesis of schizophrenia is further supported by animal studies. Mice treated with L-methionine exhibit hypermethylation of the reelin promoter and downregulation of reelin and GAD67 mRNAs and proteins [69]. In addition, these mice show schizophrenia-like behavioral alterations, including deficits in prepulse inhibition of startle and social interaction [69]. The methionine-induced mouse model was also used to examine the potential of epigenetic treatments in schizophrenia. For example, valproic acid (VPA) is a mood stabilizer that has been shown to act as a non-selective HDAC inhibitor at clinically relevant doses [70]. VPA has also been used as an adjuvant treatment for schizophrenia and has been shown to increase the clinical efficacy of atypical antipsychotic drugs, including clozapine, olanzapine, and risperidone [71,72]. Importantly, in this mouse model, VPA treatment was able to correct methionine-induced reelin promoter hypermethylation, reelin mRNA downregulation, and behavioral alterations [73]. In addition, clinically relevant doses of

the atypical antipsychotic drugs clozapine and sulpiride are able to induce demethylation of the reelin and GAD67 promoters and to potentiate the effects of VPA in the brain of methionine-treated mice [74]. All of these findings suggest that the drugs with DNA demethylating activity may be useful in the treatment of schizophrenia.

Although DNMT inhibitors have not been used in the animal models of schizophrenia, other *in vitro* and *in vivo* studies have shown that these drugs may be beneficial in treating this disorder [40,75,76]. We have found that DNMT inhibitors—doxorubicin, 5-aza-2′-deoxycytidine, and zebularine—are able to coordinately induce reelin and GAD67 mRNA expression in neuronal precursor cells *in vitro* [76]. These effects were associated with the downregulation of DNMT proteins, decreased methylation of the reelin and GAD67 promoters, and reduced association of DNMT proteins with these promoters [40]. Findings from Miller and Sweatt have shown that DNMT inhibitors are able to induce the reelin gene in the adult mouse brain. Direct infusion of DNMT inhibitors into the hippocampus of adult animals induced reelin mRNA expression, which inversely correlated with the methylation status of the reelin promoter in this brain region [47]. Together, these findings imply that DNMT inhibitors may be useful in correcting GABAergic deficits associated with schizophrenia.

In summary, there is solid evidence that DNA methylation is involved in the pathophysiology of schizophrenia. Also, findings presented in this review strongly suggest that, together with the HDAC inhibitors, DNMT inhibitors may represent a promising new therapeutic option to treat schizophrenia patients [3,77].

Therapeutic potential of DNMT inhibitors in depression

Depression is one of the most common and serious psychiatric disorders with the potential to become the leading cause of disability worldwide. Currently used antidepressants work by facilitating monoaminergic, mainly serotonergic and noradrenergic, neurotransmission [1]. Although these drugs are efficacious in relieving the symptoms, it usually takes 4–6 weeks for them to become fully effective, and more than one third of depressed patients are either partial responders or treatment resistant; thus, there is a great need for new faster-acting and more efficacious antidepressant drugs. Increasing evidence suggests that aberrant transcriptional and, in particular, epigenetic regulation is one of the key components in the pathophysiology of depression [78], providing a new avenue to search for possible treatments. So far, the main focus in animal models of depression has been on histone modifications and HDACs as possible new drug targets to treat this disorder [79]. However, mounting evidence suggests that DNA methylation may be dysregulated in depression and that DNMT dysfunction may be associated with this disorder [79,80].

Dysregulated expression of DNMTs has been found in depressed patients and in stress-induced animal models of depression. DNMT mRNA levels were altered in several brains regions of depressed suicide victims, including the frontopolar cortex, amygdala, and paraventricular nucleus of the hypothalamus [81]. Within the frontopolar cortex, DNMT3B upregulation was associated with a hypermethylation of the $GABA_A$ receptor 1 subunit promoter region, providing a possible mechanism for the downregulation of $GABA_A$ expression in suicide completers. DNMT overexpression may also be responsible for hypermethylation and consequent downregulation of other genes that may contribute to depression, such as tropomyosin-related kinase B (TrkB) that encodes the receptor for brain-derived neurotrophic factor (BDNF). Studies have shown that BDNF, as well as

TrkB, levels are decreased in suicide victims, as well as in the serum of depressed patients [82,83]. In addition, a recent study has found decreased expression of DNMT1 and increased expression of DNMT3B mRNA in the peripheral white blood cells of the patients with major depressive disorder [84]. Although there is no evidence that DNMT expression in peripheral blood cells is correlated with that in the brain, this study further implies that the DNA methylation machinery may be impaired in depression and may contribute to dysregulated gene expression. In agreement with the human data, animal studies that used different paradigms to induce anxiety- or depressive-like behavior in rodents have shown associated changes in DNMT expression. For example, low quality of maternal care increases DNMT1 expression in the hippocampus [85], and chronic stress in adulthood increases the expression of DNMT1 and 3A in the ventral striatum [31,36].

There is also evidence suggesting that the modulation of DNMT activity or DNMT expression may contribute to antidepressant activity. For instance, currently used antidepressant drugs have been shown to affect DNMT enzymatic activity *in vitro*. Therapeutic plasma levels of tricyclic antidepressants amitriptyline and imipramine, as well as the selective serotonin reuptake inhibitor (SSRI) paroxetine, have been found to reduce DNMT1 enzymatic activity in rat primary astrocytes [86,87]. *In vitro* evidence is further supported by *in vivo* studies. Melas et al. [88] and Svenningsson et al. [89] examined the effect of the SSRI escitalopram on the epigenetic regulation of *PP1*, which has been implicated in depression. Using a genetic model of depression, they were able to show that escitalopram induces hypomethylation and increased expression of *PP1* that was associated with the downregulation of *Dnmt1* and *Dnmt3a* gene expression in the prefrontal cortex of adult rats [88]. Together, these findings suggest that the efficacy of currently used antidepressants may, at least in part, involve their effect on DNMT function.

Finally, although DNMT inhibitors have not been examined in clinical trials, experimental use of DNMT inhibitors in animal models of depression suggests that these drugs may provide a new possible therapeutic option for depressed patients. For example, Sales et al. [90] have shown that systemic as well as direct hippocampal administration of DNMT inhibitors (5-azacytidine, 5-aza-2′-deoxycytidine, and RG108) induces an antidepressant-like effect in rat and mouse models of depression, similar to that observed with the antidepressant imipramine. Importantly, these effects were associated with decreased global DNA methylation and increased BDNF protein levels in the hippocampus. Decreased expression of BDNF has been associated with depression, but induction of BDNF levels is linked to antidepressant efficacy [78]. In addition, LaPlant and colleagues [31] examined whether DNMT inhibitors may attenuate depressive-like behaviors induced by stressful life events, such as chronic social defeat stress, in adulthood. This study showed that the DNMT inhibitor RG108, infused directly into nucleus accumbens (NAc), reversed stress-induced social avoidance in mice, an effect equivalent to that seen with chronic systemic treatment with the antidepressant fluoxetine [31]. Finally, depressive-like behaviors induced by chronic ultra-mild stress exposure in adult Balb/C mice were reversed by treatment with the DNMT inhibitors zebularine and RG108, which were continuously delivered into the NAc by an osmotic pump [36]. At the same time, these treatments were able to reverse stress-induced increased mRNA expression of DNMT1 and DNMT3B as well as to normalize the expression of *Gdnf* shown to modulate susceptibility and adaptation to stressful events [36].

In summary, mounting evidence suggests that epigenetic dysregulation is one of the hallmarks of depression. Although the emphasis has been on histone modifications and HDAC inhibitors as a possible new therapeutic option to treat this disorder, there is now sufficient evidence implying that modulating DNA methylation and DNMT function can provide another viable therapeutic option.

Therapeutic potential of DNMT inhibitors in bipolar disorders

Studies that have shown changes in DNMTs and altered DNA methylation levels in psychotic patients often have included bipolar patients in addition to patients with schizophrenia [61,91–93]. Therefore, aberrant DNA methylation has also been implicated in the etiology of bipolar disorder. Obviously, it is very difficult to study bipolar disorder in animal models; however, regardless of this limitation, some mechanistic studies have suggested that DNMT inhibitors could be beneficial in the treatment of this disorder. As previously mentioned, the effective mood stabilizer VPA has been shown to exert DNA demethylation effect both *in vitro* and *in vivo* [38,39], and it is possible that this effect contributes to its efficacy in treating bipolar patients. The mechanism through which VPA induces DNA demethylation is not yet clear, but it has been proposed that this drug may either directly induce critical components of the DNA demethylation machinery (which is yet to be fully characterized) or may lead to an indirect recruitment of a demethylation mechanism via its effect on HDACs and histone acetylation [71]. Interestingly, using neuronal precursor cells, we have shown that VPA reduces DNMT enzymatic activity, and this is associated with decreased DNMT expression at both mRNA and protein level [40]. Therefore, VPA may also induce DNA methylation changes by affecting the transcriptional activity of the genes that encode DNMTs. Overall, there is limited evidence suggesting that DNMT inhibitors may be useful agents in the therapy of bipolar patients; however, clinical studies will be required to assess the potential of these drugs in the clinical setting.

Therapeutic potential of DNMT inhibitors in posttraumatic stress disorder

Posttraumatic stress disorder (PTSD) is a debilitating anxiety disorder that develops in a subset of individuals who experience emotional trauma [94]. The biological basis of the development of PTSD is not known, but recent evidence suggests that epigenetic mechanisms may be involved in the pathophysiology of this disorder [9,95]. For example, a study by Smith et al. [96] has shown DNA methylation changes in immune-system-related genes in the peripheral blood samples of PTSD patients. Although the evidence of epigenetic changes in the brains of PTSD patients is limited, animal studies of fear conditioning in adult rodents strongly suggest that epigenetic mechanisms underlie fear learning, which is strongly implicated in PTSD [9].

In addition, human literature shows that a history of child abuse and maltreatment increases the likelihood of being exposed to traumatic events and of developing PTSD as an adult [95]. In line with the epigenetic hypothesis of PTSD, animal studies have shown that early-life stress can induce enduring epigenetic changes in stress-related genes and that this mediates increased stress sensitivity in adulthood [97]. Early-life maltreatment can also induce lasting changes in DNA methylation and mRNA expression of cortical *Bdnf*, which is one of the key players in learning and memory and has been shown to be reduced in PTSD [98]. In rodent models of PTSD, exposure to traumatic stimuli in adulthood is also able to change *Bdnf* expression, and this effect persists long after the fear-inducing stimulus has passed [99,100]. These findings imply that trauma-induced epigenetic changes in *Bdnf* and related genes may underlie the persistence of pathological fear and cognitive deficits observed in PTSD patients. And, because administration of DNMT inhibitors in adulthood has been shown to reverse the effect of early-life stress on *BDNF* gene expression [98], DNMT inhibitors have also been proposed as a possible new pharmacological therapy for PTSD patients [9]. However, further preclinical studies as well as clinical studies are necessary to evaluate the therapeutic potential of these compounds.

Conclusions, future directions, and challenges

The evidence summarized in this chapter strongly suggests that aberrant DNA methylation may contribute to the pathophysiology of several psychiatric disorders and that DNMT inhibitors may represent potential new therapeutics to treat schizophrenia, depression, bipolar disorders, and PTSD. Although epigenetic therapy would provide a fundamentally new way to treat these disorders, the prospect of which is highly exciting and promising, there are issues that must be addressed before these drugs can be used in the clinical setting.

One of the major concerns that is associated with the use of epigenetic drugs in the clinical setting is their lack of specificity. These drugs affect the major machinery that controls gene expression; therefore, numerous genes may be affected, resulting in high toxicity in multiple tissues including the brain. Although DNMT inhibitors have been successfully implemented in the therapy of certain types of cancers, the criteria for side-effect profiles are very different for drugs used in cancer versus those used in psychiatric disorders. Psychiatric patients usually require chronic and often life-long therapy, and they show low compliance with the therapy that is associated with severe side effects. Interestingly, studies in cancer research have shown that epigenetic drugs affect only a small subset of genes and that they primarily target genes that are epigenetically dysregulated in relation to disease. Whether this will also be the case in brain disorders remains to be determined. In addition, drugs that directly inhibit enzymatic activity of DNMTs, such as RG108, seem to be associated with generally low toxicity. Therefore, to improve specificity and toxicity profiles of DNMT inhibitors, it will be important to develop direct enzyme inhibitors that preferably target specific members of the DNMT family. For this, additional research on the role of specific DNMTs in the brain under physiological and pathological conditions will be required to inform drug design.

Another limitation for the use of drugs in treating brain disorders is the requirement of crossing the blood–brain barrier. Some DNMT inhibitors, such as 5-aza-2′-deoxycytidine and zebularine, have been administered systemically and showed epigenetic effects in the brain. However, cytidine analogs are generally highly toxic, so newly developed DNMT inhibitors will have to be both less toxic and able to cross the blood–brain barrier in order to be assessed for possible use in psychiatric disorders.

Finally, psychiatric disorders are generally very complex, involving different groups of symptoms, and it is likely that multiple mechanisms may underlie these disorders. To be able to predict which patients may respond to epigenetic drugs, we must better understand how epigenetic mechanisms contribute to specific disease phenotypes. In addition, we need to establish epigenetic biomarkers that will enable us to assess epigenetic dysregulation in connection to a specific (endo)phenotype in a particular patient. That said, it is possible that in some psychiatric disorders epigenetic drugs will have to be combined with other drugs or with behavioral therapy in order to control different facets of these disorders.

In summary, although DNMT inhibitors represent a promising new therapeutic option for several psychiatric disorders, significant basic and clinical research is necessary before these drugs are ready for clinical use.

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Glossary

Cytidine A nucleoside formed by the condensation of cytosine and the sugar ribose.
Cytosine A pyrimidine base that is one of the fundamental components of DNA and RNA.
In silico Performed on computer or by computer simulation.
Myelodysplastic syndromes Hematological conditions where stem cells of bone marrow do not mature into healthy blood cells.

CHAPTER

Histone Deacetylase Inhibitors and Psychiatric Disorders

25

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KEY CONCEPT

- Histone deacetylase inhibitors may prove to be beneficial in the treatment of patients with neuropsychiatric disorders.

ABBREVIATIONS

AD Alzheimer's disease
ALS Amyotrophic lateral sclerosis
BBB Blood–brain barrier
BDNF Brain-derived neurotrophic factor
BPD Bipolar disorder
CeA Central amygdala
CHAPs Cyclic hydroxamic acid-containing peptides
ChIP-chip Chromatin immunoprecipitation coupled with promoter microarray
CNS Central nervous system
CTCL Cutaneous T-cell lymphoma
CUMS Chronic unpredictable multiple stress
DRPLA Dentatorubral–pallidoluysian atrophy
H3K9 Histone H3 lysine 9
HAT Histone acetyltransferase
HD Huntington's disease
HDAC Histone deacetylase
HDACi Histone deacetylase inhibitors
HRM Heterozygous *reeler* mouse
LTP Long-term potentiation
MDD Major depressive disorder
MeA Medial amygdala
NAc Nucleus accumbens
NAD^+ Nicotinamide adenine dinucleotide
NPY Neuropeptide Y
PB Phenylbutyrate
PD Parkinson's disease
PFC Prefrontal cortex
PTSD Posttraumatic stress disorder
ROCK1 Rho kinase 1
SAHA Suberoylanilide hydroxamic acid
SCFAs Short-chain fatty acids
SGZ Subgranular zone
SIRT Sirtuin
SSRI Selective serotonin reuptake inhibitor
SZ Schizophrenia
TSA Trichostatin A
vSTR Ventral striatum

Introduction

The molecular mechanisms underlying the etiopathology of complex disorders such as cancer, metabolic disorders, and disorders of brain and behavior are still elusive. However, recent research efforts into epigenetic or chromatin remodeling mechanisms that control transcription have been quite promising, leading us to devise new therapeutic strategies to treat complex disorders, including neuropsychiatric disorders [1–7]. The epigenetic regulatory mechanisms involve myriad events such as DNA modifications and DNA-bound histone and non-histone proteins, changes in chromatin conformation in and around genes, and regulating gene function [8]. Chronic environmental perturbations such as stress or abusive drugs have been shown to dysregulate signal transduction pathways in neuronal and glial cells, which in turn alter the activity of specific transcription regulators, resulting in alterations in chromatin conformation in regulatory regions

of many genes, ultimately affecting the level of gene transcription [9,10]. Thus, perturbation-induced changes in chromatin and gene activities affect the behavior of cells or tissues, resulting in the development of disease states [11]. However, the innumerable epigenetic regulatory mechanisms that control gene expression, such as DNA methylation/demethylation, histone acetylation/deacetylation, methylation/demethylation, phosphorylation, and ubiquitylation, among the many modifications, are yet to be completely understood [2,8]. Of these chromatin remodeling mechanisms, histone acetylation and deacetylation are better understood. The proteins or enzymes responsible for histone acetylation and deacetylation are known as histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively [12]. HATs add acetyl groups at histone lysine residues; this increase in acetylation helps in chromatin loosening (euchromatin) and thus activates transcription. In contrast, HDACs remove acetyl groups on histones H3 and H4 lysine (K) residues, causing chromatin conformational changes around active genes which become more compact (heterochromatin), hence repressing gene transcription [13].

In most neuropsychiatric disorders, histone acetylation has been found to be downregulated in specific brain areas implicated, due to higher activity of HDACs [14]. Various research groups have administered HDAC inhibitors (HDACi) to a number of animal models of neuropsychiatric disorders and found that these inhibitors ameliorate disease conditions [14,15]. However, because all HDACi tested in the last decade are non-specific, inhibiting multiple Zn^{2+}-dependent HDAC isoforms (pan inhibitors), the focus now has been on developing isoform-selective inhibitors. Moreover, the role of various HDAC members in normal and pathological brain is not completely understood.

HATs and HDACs maintain transcription homeostasis

Histones are basic proteins that provide a scaffold to wrap and condense DNA in the nucleus. The binding of histones to DNA is altered by acetylation or deacetylation of histone lysine residues, which determines the accessibility of the regulatory regions of genes to transcription factors facilitating transcription. This dynamic equilibrium state of acetylation and deacetylation at particular genomic loci is maintained by the interplay of spatially and temporally regulated HATs and HDACs, respectively (Figure 25.1). HATs, by acetylating histone lysine residues (Figure 25.1), impart effective negative charges to histones, which results in increased electrostatic repulsion between histones and DNA. This helps the chromatin to unwind to provide transcription factor accessibility at the regulatory regions of target genes to initiate transcription. Similarly, HDACs remove the acetyl groups at histone lysine residues (Figure 25.1) causing tightening of the chromatin around gene-regulatory regions and resulting in the repression of transcription. Histone acetylation and deacetylation thus control most physiological and pathological cellular processes, including those in the brain, by regulating transcription of genes [16].

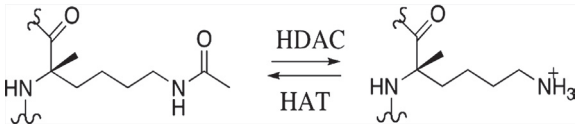

FIGURE 25.1

The roles of HAT (transcription activation) and HDAC (transcription repression).

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HDACs

Histone deacetylase proteins are involved in the regulation of gene expression by causing the deacetylation mostly of lysine residues at N-terminal tails of the histone protein, resulting in the inaccessibility of the regulatory regions by the transcription factors, a critical step for transcription initiation. Hence, HDACs function as repressors or gene silencers of transcription and are implicated in the etiopathology of a number of neuropsychiatric disorders. The RNA and/or protein levels of different HDAC members have been shown to be upregulated in different parts of the brain, resulting in dysregulation of many cell signaling pathways [17]. Upon treatment with HDACi such as valproate, butyrate, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA), disease symptoms improve in animal models of neuropsychiatric disorders due to the restoration of the dysregulated cellular signaling [18]. Mounting evidence shows the dysregulation of one or more HDACs in psychiatric disorders such as depression, anxiety, schizophrenia, and bipolar disorder [1]. A comprehensive understanding of the role of HDACs in psychiatric disorders and the design of rational inhibitors of HDACs may provide therapeutic alternatives for treating these complex disorders.

HDAC types

Histone deacetylase enzymes comprise a large family of proteins. The 18 HDACs known thus far in humans are grouped in four classes. Class I is comprised of HDAC1, 2, 3, and 8, which reside mainly in the nucleus [19,20]. Class IIa includes HDAC4, 5, 7, and 9, and Class IIb consists of HDAC6 and 10. Class IIb enzymes have the unique characteristic of having two deacetylase domains. Class II enzymes can shuttle back and forth between nucleus and cytoplasm in response to a wide variety of cellular signals [21–23]. HDAC11 is the sole representative of Class IV and has the same characteristics of Class I and II enzymes [20], but it is thought to have different physiological functions. Unlike Class I, II, and IV HDACs, which are Zn^{2+}-dependent enzymes, Class III HDACs require nicotinamide adenine dinucleotide (NAD^+) for their activity and are named sirtuins (SIRTs 1–7) [24–27]. Here, we will discuss classical HDACs (Zn^{2+}-dependent) and the HDACi acting on these Zn-dependent HDACs rather than on sirtuins, as the neurobiology of these NAD^+-dependent Class III HDACs is not yet clear. The functions of Zn^{2+}-dependent HDACs in the context of brain and behavior are summarized in Table 25.1.

HDACs in normal brain, behavior and in psychopathology

HDAC class I

At the mRNA level, HDAC1, along with HDAC2 and 3, exhibits high levels of expression in cerebellar granule neurons, whereas HDAC3 shows quite prominent levels of expression, followed by HDAC2, in the striatum (see the references in Table 25.1). The hippocampus presents a complex scenario, as all of the classical HDAC enzymes show some level of expression but with differential brain subregion-specific expression: HDAC1 and 8 show greater mRNA expression in the neurogenic dentate gyrus compared with the CA1 and CA3 regions. At the global level, it is HDAC2 that shows the highest level of expression compared with other HDACs in the hippocampus (see references in Table 25.1). Modulation of HDAC2 in mice by overexpression or knockout strategies results in impaired long-term potentiation and

Table 25.1 Biology of HDACs in Models of Neuropsychiatric Disorders

Class	Type (Subcellular Localization)	Biological Functions/Implication in Brain Diseases	References
Class I	HDAC1 (nucleus)	HDAC1 is significantly increased in prefrontal cortex, hippocampal neuronal layers, and median temporal lobe in schizophrenia patients.	Sharma et al. [28]
		HDAC1 expression is decreased in hippocampus in bipolar disorder.	Benes et al. [29]
		In hypoxia, HDAC1 is increased in neuronal cultures. Loss of HDAC1 accelerates neurodegeneration, and gain of function ameliorates ischemia-induced neuronal death.	Wang et al. [30]
		HDAC1 facilitates memory extinction by increasing H3K9 acetylation at c-*fos* promoter. HDAC1 level is increased in hippocampus after contextual fear conditioning.	Bahari-Javan et al. [31]
	HDAC2 (nucleus)	Overexpression of HDAC2 in frontal cortex in a mouse model resulted in a schizophrenia-like phenotype.	Kurita et al. [32]
		HDAC2 is a negative regulator of memory formation in mice.	Guan et al. [33]
		HDAC2 constrains LTP and associative and spatial memories in healthy and neurodegenerated mouse brain; it is decreased after contextual fear conditioning.	Gräff et al. [34]
	HDAC3 (nucleus/ cytoplasm)	HDAC3 is a critical negative regulator of long-term memory formation and affects spatial memory; it is decreased after contextual fear conditioning.	McQuown et al. [35]
		HDAC3 mediates neurotoxicity in HD model.	Bates et al. [36]
	HDAC8 (nucleus/ cytoplasm)	HDAC8 is a negative regulator of CREB-dependent transcription.	Gao et al. [37]
Class IIa	HDAC4 (nucleus/ cytoplasm)	Deletion of HDAC4 improves thermotaxic memory in *C. elegans*.	Wang et al. [38]
		Loss of HDAC4 impairs LTP and associative and spatial memories. HDAC4 regulates neuronal plasticity linked to addiction.	Kim et al. [39]
	HDAC5 (nucleus/ cytoplasm)	HDAC5 regulates neuronal plasticity linked to addiction, mediates antidepressant effects, and decreases long-term facilitation by binding to the promoter region of C/EBP.	Renthal et al. [40]
		HDAC5 is increased in the mouse model of schizophrenia with memory deficits.	Guan et al. [41] Tasankova et al. [42]
		HDAC5 is decreased after contextual fear conditioning.	Koseki et al. [43]
	HDAC7 (nucleus/ cytoplasm)	Loss of HDAC7 does not affect pathogenesis of HD; it is decreased after contextual fear conditioning.	Benn et al. [44]
	HDAC9 (nucleus)	Copy number variation in HDAC9 is associated with schizophrenia in humans.	Tam et al. [45]
		HDAC9 is neuroprotective and promotes dendritic growth.	Zhang et al. [46]

(*Continued*)

Table 25.1 (Continued)

Class	Type (Subcellular Localization)	Biological Functions/Implication in Brain Diseases	References
Class IIb	HDAC6 (cytoplasm)	HDAC6 acts as a microtubule deacetylase; it is expressed in most neurons but is most abundant in cerebellar Purkinje cells.	Hubbert et al. [47] Pandey et al. [48]
		Neuroprotective or neurodegenerative effects are observed, depending on the model system.	Rivieccio et al. [49] Simões-Pires et al. [50]
	HDAC10 (nucleus/cytoplasm)	Unknown.	—
Class IV	HDAC11 (nucleus)	HDAC11 regulates oligodendrocyte-specific gene expression and oligodendrocyte development; it is increased after contextual fear conditioning.	Liu et al. [51]

enhanced neuroplasticity with improved cognition. The cognitive enhancement in the HDAC2 knockout mouse has been shown to be associated with increased hippocampal histone acetylation, specifically at the promoter region of genes involved in synaptic plasticity [33]. Local deletion of HDAC3 in the mouse hippocampus also increases hippocampal histone acetylation and, as such, establishes its role in learning and memory [35]. The interplay of HDACs in brain processes is complex due to the differential roles of various HDACs in the same process. HDAC2 and HDAC3 downregulation in the hippo-campus of fear-conditioned rats after learning is associated with the upregulation of HDAC1. Recent investigations show significantly high levels of HDAC1, together with high HDAC3 levels, in both prefrontal cortex (PFC) and hippocampus in schizophrenia and bipolar patients, which exhibit a negative correlation with the GAD67 transcript level (critical in GABAergic signaling) [29]. Increased levels of HDAC1 in the hippocampus and the consequent decrease in the H3K9 acetylation level at the c-*fos* promoter was found in contextual fear extinction learning and memory [31]. In hypoxia, the H3K9 acetylation is lower due to increased levels of HDAC1, thus HDAC1 has been implicated in hypoxia-induced amyloid plaque formation [30]. Significantly higher levels of HDAC2 were found in the PFC of schizophrenia patients, which appeared to decrease the acetylation of histones at the mGlu2 promoter [32]. The levels of HDAC2 were decreased in contextual fear conditioning. HDAC3 inhibition by an HDACi, RGFP966, resulted in the extinction of cocaine-seeking behavior [35] by enhancing long-term object memory through differential gene expression in the hippocampus and nucleus accumbens (NAc), and HDAC3 is also involved in negative regulation of memory in the hippocampus [35].

HDAC class IIa

Among Class IIa HDACs, HDAC5 is highly expressed in the striatum and hippocampus, and HDAC4 expression is prominent in the hippocampus only. Due to lower basal HDAC activity, members of this class, especially HDAC4, 5, and 9, are thought to indirectly act on non-histone substrates. Haploinsufficiency of HDAC4 causes brachydactyly mental retardation syndrome, developmental delays, and behavioral problems. Brain-specific deletion of HDAC4 impairs long-term potentiation

(LTP) and memory in mice and nematodes [38,39]. It also has been shown to play a role in inhibiting cell-cycle progression and in preventing neuronal cell apoptosis. HDAC5 overexpression and concurrent reduction of H3 acetylation in the PFC have been shown to be associated with impaired object recognition memory in a mouse model of schizophrenia [43]. Also, HDAC5 and HDAC7 are downregulated in the hippocampus of rats after fear-conditioning training. A recent report [42] implicated HDAC5 in the hippocampus in social-defeat-induced depression-like conditions in mice. Imipramine's antidepressant activity in this model was shown by the enhanced histone H3 acetylation at K9 and 14 at BDNF promoters III and IV by downregulating HDAC5. The overexpression of HDAC5 in the hippocampus, using herpes simplex virus-mediated gene targeting, blocks the antidepressant action of imipramine [42]. Although the role of HDAC9 is not as well defined in the brain, a few recent investigations show HDAC9 copy number variations in patients with schizophrenia who had cognitive disabilities and in healthy control subjects.

HDAC class IIb

Among the Class IIb HDACs, HDAC6 has been shown to be expressed in brain mostly in cerebellar Purkinje cells. Recent evidence shows that HDAC6 has been part of the microtubule network where it acts at a specific α-tubulin deacetylase. HDAC6 is also involved in ubiquitin–proteosome and lysosomal autophagy-mediated protein aggregate degradation, which is the principal factor for the initiation of many neurodegenerative disorders such as Parkinson's disease (PD) (Lewy bodies) and Alzheimer's disease (AD) (amyloid plaques). HDAC6 appears to be a target in neurodegenerative disorders, and its inhibitor tubacin has potential as a therapeutic alternative [48–50].

HDAC class III

Despite the critical role of sirtuins in neuronal function, not much is known regarding the role of sirtuins in brain and behavior and their implication in neuropathological disorders. Out of seven NAD^+-dependent enzymes [52], only SIRT1 and SIRT2 have been studied in the brain in relation to neurodegenerative disorders such as AD, PD, and Huntington's disease (HD). SIRT1 has been shown to protect neurons against amyloid-beta (Aβ)-induced toxicity by inhibiting NF-κB signaling in microglia or by downregulating the serine/threonine ROCK1 (Rho kinase 1) in neurons [53]. SIRT1 overexpression or treatment with the sirtuin activator resveratrol mitigates the progression of neurodegenerative disorders. SIRT2 inhibition in the *Drosophila* model of PD and in primary dopaminergic cultures helps to rescue α-synuclein-mediated cytotoxicity [53]. Recently, it was shown that SIRT1 and SIRT2 inhibition results in less cocaine intake in the mouse cocaine self-administration model [54]. SIRT1, 2, and 6 levels have been shown to be significantly reduced in peripheral white blood cells in mood disorder patients [55]. Most of the neurobiological investigations of SIRT1 and SIRT2 using animal models suggest that activation of SIRT1 and inhibition of SIRT2 might have therapeutic potential in treating a number of neuropsychiatric disorders.

HDAC class IV

The role of HDAC11 in the adult brain remains to be investigated.

HDAC inhibitors

A number of small-molecule HDACi and *in vivo* studies using animal models of cancer, inflammatory diseases, metabolic disorders, and neurodegenerative, neurological, and psychiatric disorders suggest their potential for the treatment of these complex disorders [1,5,13–15,18]. HDACi developed in recent decades belong to various classes and each member of a particular class has a different functional group with the HDAC inhibitory activity. However, each of these HDACi compounds blocks the activity of one or more of the 11 HDACs, resulting in increased histone lysine acetylation on histones, as well as increased acetylation of a number of non-histone proteins, such as transcription factors, tumor suppressors, etc. [1]. The administration of HDACi to a number of animal models of neurological and psychiatric disorders has been shown to open condensed chromatin around the promoter regions of many key genes such as *BDNF*, which is often repressed in the diseased state, resulting in the de-repression of expression and amelioration of the disease phenotype [18]. The efficacy of various HDACi, such as butyrate and valproate (short-chain fatty acid class), TSA, SAHA (hydroxamate class), MS-275 (benzamide class), trapoxin, and apicidin (cyclic tetrapeptide class), had been investigated successfully in preclinical models of non-central nervous system (CNS) disorders, as well as CNS disorders, before a number of these entered into clinical trials [1,5,11,14,15,18,56].

Recent analyses of clinical trial data suggest that a number of these HDACi are in the first two phases of clinical trials. However, out of all the HDACi trials against non-CNS diseases most of these are for various types of cancers (as shown in Figure 25.2). Except for two clinically approved drugs, vorinostat (SAHA) and panbinostat, for treatment of cutaneous T-cell lymphoma, not many of the HDACi have entered into Phase III clinical trials even against cancer (Figure 25.2) [18]. The same is true of the chemical agents that act on chromatin regulatory mechanisms other than HDACs, as shown in Figure 25.2. This appears to be due to the fact that most of these are pan inhibitors which have adverse effects due to lack of specificity on the target. These initial trials for HDACi were

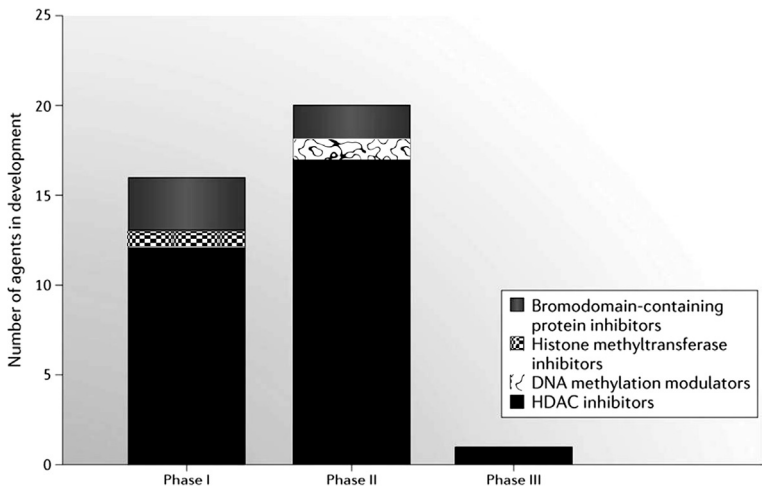


FIGURE 25.2

The trend of clinical trials for HDACi in non-CNS and CNS diseases.

conducted primarily on cancer patients because: (1) the role of histone acetylation and deacetylation is better understood in oncogenesis, and (2) the treatment of terminal diseases such as cancer is a priority. Although few of the HDACi are in clinical trials for neurological disorders, preliminary review of the initial phase of these trials gives reason for optimism.

Clinical trials of HDACi

The clinical trial outcome for the new HDACi molecules RGF2833 and EVP-0334 on neurodegenerative disorders (see Table 25.2) seems optimistic. However, it must be noted that most of the available compounds working as HDACi are pan-HDAC inhibitors and so lack specific targets of action.

HDACi tested in animal models of neuropsychiatric disorders

The outcomes of investigations into the efficacy of HDACi belonging to different classes on rodent models of neurological and psychiatric disorders suggest their therapeutic potential. Almost all animal models for CNS diseases have shown significant improvements upon the administration of HDACi such as valproate, butyrate, TSA, SAHA, and MS-275 (see references in Table 25.3).

HDACi in psychotic disorders (e.g., schizophrenia, bipolar disorder)

Neuropathology and molecular genetic mechanisms of complex neuropsychiatric disorders like schizophrenia (SZ) and bipolar disorder (BPD) remain poorly understood. Recent research efforts in this direction have highlighted novel and precise drug targets such as HDACs, as mentioned in the previous section, which raised high expectations for the use of HDACi to treat neuropsychiatric conditions, particularly SZ. It is now becoming clear that many antipsychotic drugs work at the chromatin level and change posttranslational histone modifications such as acetylation and methylation [1,8]. Subcutaneous injection of valproic acid, a Class 1 HDAC inhibitor, significantly increases H3 histone acetylation in frontal cortex 2 hours after injection in heterozygous *reeler* mouse (HRM) mice and in the methionine-induced hypermethylation model of SZ, concurrently increasing the expression of reelin and GAD67, which have been reported to be repressed in brains of SZ patients [68]. SZ is associated with impaired fear extinction, and it has been shown that valproate administration before fear extinction training rescues impaired fear extinction in 129S1/SvImJ S1 mouse, a model of impaired fear extinction [66]. Valproic acid also attenuates the mania-like behavior in the dopamine transporter knockdown mouse model of SZ and the pharmacologic SZ mouse model (GBR12909) to a large extent [69]. Non-specific HDACi in combination with currently available antipsychotics, particularly second-generation antipsychotics (usually prescribed when the first line of drugs fails to evoke positive response in patients), hold great promise to treat SZ and other psychotic disorders. Long-term treatment with clozapine, a second-generation antipsychotic, is detrimental as it induces compensatory overexpression of HDAC2 in the frontal cortex, as mentioned earlier, which in turn causes the downregulation of metabotropic glutamate receptors 2 and 3. However, co-administration of SAHA with clozapine greatly improves therapeutic efficacy in male 129S6/SvEv mice, in addition to potentiating the antipsychotic effects of LY379268, a metabotropic glutamate receptor agonist [78]. However, all of the above-mentioned compounds act as

Table 25.2 Clinical Trials for HDACi in Non-CNS and CNS Diseases

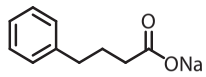
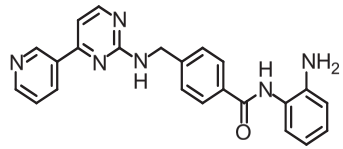
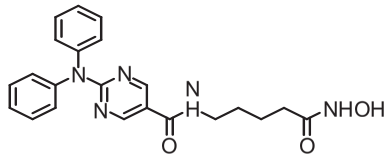
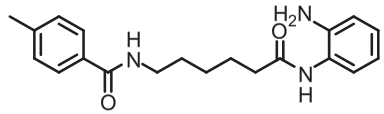
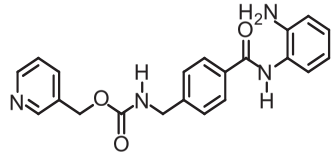
Phase	Drug/agent	Structure of Drug/agent	Target/mechanism of action	Potency
Huntington's disease (I), amyotrophic lateral sclerosis (I/II)	Sodium phenylbutyrate		HDAC Class I, IIA inhibitor	m*M*
Huntington's disease	SEN0014196	—	SIRT1	—
Neurodegenerative disorders (I)	EVP-0334	—	Class I HDAC inhibitor	—
Diffuse large B-cell lymphoma (II), follicular lymphoma (II)	Mocetinostat		Class I HDAC inhibitor	μm
Multiple myeloma (I/II)	ACY-1215 (rocilinostat)		HDAC6 inhibitor	5 n*M*
Neurodegenerative disorders (Friedreich's ataxia)	RG2833 (available orally)		Class I HDAC (HDAC3)	—
AIDS (I/II), Hodgkin's lymphoma (III), cutaneous T-cell lymphoma (II)	Panobinostat	—	HDAC inhibitor (HDAC1, 2, 3)	μm
Non-small-cell lung cancer (II), Hodgkin's lymphoma (II), breast cancer (II), acute myeloid leukemia (I)	Entinostat (MS-275)		Class I HDAC inhibitor	300 n*M* (HDAC1) 8 μm (HDAC3)

Table 25.3 Efficacy of HDACi in Animal Models of Major Psychiatric Disorders

Structural Class	HDACi	Selectivity	Animal Model	Implications in Psychiatric Disorders	Refs.
Carboxylic acids	Sodium butyrate (NaB)	Classes I, IIa	Permanent MCAO cerebral stroke model	Induced proliferation of neural stem cells in DG and SVZ, striatum and prefrontal cortex; increased the number of cells expressing polysialic acid–neural cell adhesion molecule, nestin, glial fibrillary acidic protein, phospho-cAMP response element-binding protein (CREB), and BDNF in various brain regions after cerebral ischemia.	Kim et al. [57]
			Defeat-induced depression mouse model	Ameliorated anxiety and restores normal mood.	Schroeder et al. [58]
			Addiction	Reversed ethanol-induced behavior sensitization in DBA/2J mice model by modulating the gene expression alteration in stratum.	Legastelois et al. [59]
				Inhibited morphin-induced hyperactivity and behavioral sensitization by increased histone acetylation in NAc of mice.	Jing et al. [60]
			Neurogenerative diseases	Ameliorated histone hypoacetylation and neurodegenerative phenotypes in dentatorubral–pallidoluysian atrophy (DRPLA) of transgenic mice model (Atro-118Q).	Ying et al. [61]
	Phenylbutyrate (PB)	Classes I, IIa	G93A transgenic ALS model	Restored histone acetylation to near-normal levels and induced expression of prosurvival factors such as pIkB, NF-κB p50, and bcl-2.	Ryu et al. [62]
			Cocaine addiction	Increased sensitivity and reduced cocaine self-administration, correlating with frontal HDAC levels in rat model.	Romieu et al. [63]
			Neurodegenerative disorders	Ameliorated cognitive deficit and reduces tau pathology in an Alzheimer's disease mouse model.	Ricobaraza et al. [64]
				Showed neuroprotective effect in N171-82Q transgenic mouse model of Huntington's disease.	Gardian et al. [65]

(*Continued*)

Table 25.3 (Continued)

Structural Class	HDACi	Selectivity	Animal Model	Implications in Psychiatric Disorders	Refs.
	Valproic acid (VPA)	Class I	129S1/SvImJ S1 mouse with no extinction learning following normal fear conditioning (schizophrenia-like impaired fear extinction)	Rescued impaired fear extinction learning and deficient extinction consolidation when administered before extinction training.	Whittle et al. [66]
				Altered gliogenic destined neural progenitors to neurogenic fate in subgranular zone (SGZ) of mouse hippocampus.	Hsieh and Gage [67]
			HRM mice/ MET-induced schizophrenia mouse mode	Injections of valproate (from 0.5–4 mmol/kg) increased acetylation of histone H3 in FC; valproate rescued downregulated expression of *reelin* and *GAD67* mRNAs (genes implicated in SZ).	Tremolizzo et al. [68]
			DAT knockdown mice model of SZ pharmacologic (GBR1) model	Partially attenuated mania-like behavior in mice.	Van Enkhuizen et al. [69]
			Disc1-L100P mutant mice (SZ-like phenotype)	Prevented the emergence of SZ-like abnormal behavior when injected in 8-week-old mice; normalized Lnc2 and GFAP expression.	Lipina et al. [70]
			Neurodegenerative disorders	Improved memory deficits and reduces the neuronal loss in APP/PS1 double transgenic Alzheimer's disease mice.	Long et al. [71]
			Unilateral carotid ligation of P12 CD1 mouse model of stroke-induced brain injury	Significantly increased dentate gyrus neurogenesis.	George et al. [72]
Hydroxamic acid	Trichostatin A (TSA)	Classes I, II	Rat model of alcohol addiction	Reduced alcohol withdrawal induced anxiety; normalized NPy expression (in amygdala).	Host et al. [73] Pandey et al. [74]

		Unilateral carotid ligation of P12 CD1 mouse model of stroke-induced brain injury	Significantly increased dentate gyrus neurogenesis.	George et al. [72]
		Cocaine addiction	Increased sensitivity and reduced cocaine self-administration correlating with frontal HDAC levels in rat model.	Romieu et al. [63] Host et al. [73]
		Neurodegeneration	Rescued synaptic plasticity and associative memories in APP/PS1 mice.	Francis et al. [75]
Tubastatin A	HDAC6	HSPB1S135F-expressing transgenic mice (Charcot–Marie–Tooth disease)	Induced α-tubulin hyperacetylation in primary cortical neuron cultures; increased total numbers of mitochondria; increased motor performance of HSPB1S135F-expressing mice neuronal protection of primary cortical neuron cultures.	d'Ydewalle et al. [76]
MC1568	Class II-specific (HDAC4, 5)	Primary cortical neuron culture	Increased BDNF level in cortical neurons.	Koppel and Timmusk [77]
SAHA (vorinostat)	Classes I, II, IV	Male129S6/SvEv mice (SZ)	Improved clozapine's therapeutic efficiency, potentiated the antipsychotic response to novel compounds such as LY379268, a metabotropic glutamate receptor agonist; increased mGlu2 and mGlu3 expression.	Kurita et al. [78]
		C57BL/6J male mice social-defeat model	Ameliorated depressive behavioral profiles through increase in H3K14 acetylation in nucleus acumens.	Covington et al. [79]
		CUMS mouse model of depression	Reversed depression phenotype in stressed mice; increased social interaction and sucrose preference; increased mRNA levels of GDNF in the vSTR; decreased immobility time in forced swim test.	Uchida et al. [80]
		Rubinstein–Taybi syndrome (CBP knockout mouse)	Enhanced synaptic plasticity; improved learning and memory.	Valor et al. [81]

(*Continued*)

Table 25.3 (Continued)

Structural Class	HDACi	Selectivity	Animal Model	Implications in Psychiatric Disorders	Refs.
			Alcohol addiction	Reduced the alcohol dependence or alcohol self-administration.	Rouaux et al. [82]
			Neurodegenerative diseases	Rescued neuronal cell death and ameliorated motor impairment in HD mouse model.	Rouaux et al. [82]
Benzamide group	MS-275	HDAC1–9	Male C57BL/6 wild-type mice (contextual fear extinction)	Intrahippocampal injection of MS-275 impaired fear extinction.	Bahari-Javan et al. [83]
			C57BL/6J male mice social defeat model	Ameliorated depressive behavioral profiles; increased H3K14 acetylation in nucleus acumens.	Covington et al. [79]
			Alcohol addiction	Reduced alcohol self-administration and seeking.	Warnault et al. [84]
	Crebinostat	HDAC1, 2, 3, 6	Male C57BL/6J mice	Increased pan histone acetylation; increased H4K12 and H3K9 acetylation in primary cortical brain neurons; increased context-dependent freezing behavior and hence memory; upregulated many CREB-regulated genes (e.g., *hsp70*, *BDNF*).	Fass et al. [85]
	CP60	HDAC1, 2	C57BL/6 AIH mouse models of depression	Systemic injection increased H2B, H3K9ac, and H4K12 acetylation in NAc and hippocampus; significantly reduced hyperlocomotion in amphetamine-induced hyperlocomotion (AIH) and immobilization time in forced swimming text; increased expression of Agxt211, Sgk1, and Tsc22d3 in NAc.	Schroeder et al. [86]
	RGFP136	HDAC3	Mouse model	Enhanced hippocampal-mediated learning and memory; extinction of cocaine-seeking behavior and cocaine-conditioned place preference by modulating the histone acetylation in infralimbic cortex, hippocampus, and nucleus accumbens.	McQuown et al. [35] Malvaez et al. [87]

pan HDACi and, as such, precise molecular mechanisms underlying the behavioral outcomes remain obscure. Moreover, in one study, use of the specific HDACi fails to evoke the same responses as shown by the intra-hippocampal injection of MS-275, an HDAC1-specific inhibitor that interferes with contextual fear extinction in male C57BL/6 mice [83]. This complexity, associated with the epigenetic heterogeneity of drug action and etiopathologies of psychiatric disorders, emerges as a major hurdle during clinical trials. Thus, the major challenge in treating psychotic disorders is to extensively explore brain-region- and cell-type-specific epigenetic mechanisms underlying these disorders, along with the development of specific HDACi to reverse such dysregulated events.

HDACi in MDD

Chronic stressful events induce conditions similar to human depression, posttraumatic stress disorder (PTSD), and related mood disorders in mouse models. Alterations in chromatin modification profiles in reward circuitry that are distinctly different from healthy control subjects have been attributed to the development of pathophysiology in major mood disorders including BD (for references see Table 25.3). Recent efforts have generated an extensive body of evidence for the association between environmentally induced (e.g., chronic exposure to stress) changes in genome-wide histone posttranslational modifications, particularly in the acetylation landscape, and regulation of gene expression, brain plasticity, and behavioral changes (for references see Table 25.3). Depression-like behavioral phenotype in the social-defeat mouse model is in part mediated by transcriptionally repressive methyl marks on histone H3K9 and H3K27 around the regulatory regions of BDNF in the hippocampus and hundreds of genes in NAc [88]. Interestingly, chronic treatment with imipramine, a common tricyclic antidepressant, restores the decrease in BDNF mRNA with concurrent stabilization of the mood by reversing the epigenetic marks on the *BDNF* promoters in the hippocampus [42] and also on a number of dysregulated promoters in NAc [89]. In another preclinical mouse model of depression, amelioration of stress-induced anxiety and restoration of mood and BDNF transcript levels were achieved by treatment with sodium butyrate, a Class I- and IIa-specific HDAC inhibitor [58–61]. Impaired neurogenesis, mediating the long-lasting changes in neuronal circuitries, has been implicated in the etiopathology of major depressive disorder (MDD) [57], and epigenetic modulation of this process by HDACs and HDACi holds great promise for treating these debilitating and chronic pathologies in the near future. Inhibition of HDACs by valproate has been shown to induce the fate of gliogenic neural progenitors to the neurogenic fate in the subgranular zone (SGZ) of mouse hippocampus [90], and valproate activates the expression of a repressed neurogenic transcription factor, NeuroD. Another nonspecific pan HDACi, SAHA, a synthetic hydroxamate, is able to reverse the depression-like behavioral phenotype, as evident by an increase in social interactions and sucrose preference and a decrease in immobility time in the forced swim test. This was because SAHA induces increases in histone H3K14 acetylation and subsequent transcriptional activation of several genes such as *GDNF* in the ventral striatum (vSTR) in social defeat and chronic unpredictable multiple stress (CUMS) mouse models of depression [78–81,84]. One of the more specific HDACi, MS-275 (entinostat), is also quite efficient in restoring normal mood in mice showing depression- and anxiety-like phenotypes in the preclinical social-defeat mouse models of mood disorders [79]. These studies provide novel leads for the future development of epigenetic-based therapeutics for mood disorders. However, it should be kept in mind that intensive research efforts are needed to obtain better insight into brain-region- and

cell-type-specific mechanisms of action of individual HDACs in neuropsychiatric pathologies before specific HDACi become a reality as therapy.

HDACi in drug addiction

Activation of the mesolimbic dopaminergic neurons in brain reward circuitry mediates the development of similar neuroglial changes and an addictive behavior phenotype upon exposure to various drugs of abuse (for references see Table 25.3). Overlapping addictive syndromes of psychoactive drugs, although with significantly different mechanisms of action, offer the emerging theme of shared molecular neuroadaptation in the reward pathway that mediates long-lasting changes underlying addiction. It is becoming increasingly clear each day that most of these adaptations are mediated through epigenetic and differential transcriptional regulation of many genes upon chronic exposure to drugs of abuse (see references in Table 25.3). Chronic or acute exposure to cocaine increases global levels of H3 and H4 acetylation in NAc or the ventral striatum, a critical center in the brain reward circuitry. Addiction-related behaviors in rodent models of addiction are severely affected by alterations in histone acetylation. Systemic or intra-NAc administration of inhibitors against Class I and II HDACs (e.g., butyrate, TSA) significantly potentiates behavioral responses to cocaine and decreases self-administration [73,84]. In contrast, viral-mediated overexpression of HDAC4 and HDAC5, but not HDAC9, in the NAc and the Class III HDAC inhibitor, sirtinol, have the opposite effect. Intraperitoneal injection of TSA restores the reductions in histone acetylation and in the mRNA and protein levels of neuropeptide Y (NPY) in the CeA and MeA, and ameliorates the alcohol withdrawal-induced anxiety in rat models of addiction [74]. SAHA and MS 275 also show positive results in controlling alcohol withdrawal-induced anxiety behaviors and in reducing alcohol intake [74].

HDACi in neurological disorders

Neurological disorders that are left untreated, or have reached a level of severity that they impact the behavior of an individual, may lead to psychiatric disorders. For this reason, it is appropriate to discuss studies using HDACi in models of neurological disorders (such as stroke) and neurodegenerative disorders. Sodium butyrate administration for 4 weeks improves the neurodegeneration-associated memory and structural plasticity impairments in the CK-p25 and APP/PS1 mouse models of AD. Phenylbutyrate injections reduce neurotoxic amyloid-β with promising *in vivo* neuroprotective potential in animal models of HD and amyotrophic lateral sclerosis (ALS); they restore histone acetylation to near-normal levels and induce expression of pro-survival factors such as pIkB, NF-κB p50, and Bcl-2 in G93A transgenic ALS mice [62,66]. Acute treatment with SAHA or sodium butyrate successfully normalizes H4K12 acetylation deficits and partially abrogates corresponding gene expression deficits. TSA and sodium butyrate also normalize long-term potentiation deficits associated with various degenerative diseases linked with age-related disorders. The investigations using HDACi from various classes in animal models of neurodegenerative and other neurological disorders, including cognitive disorders, suggest their potential in treating these disorders. However, the non-specific actions of various preclinically successful HDACi limit their efficacy and efficiency for

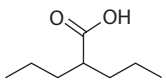
various clinical trials. Furthermore, the biological effects of manipulating acetylation levels (particularly increasing histone lysine acetylation) on other epigenetic marks in particular need to be investigated in normal unperturbed systems. Even more troublesome are the potential adverse effects of these drugs acting on targets in peripheral organs outside the brain.

HDACi classes

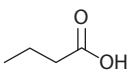
Histone deacetylase inhibitors comprise a diverse class of synthetically derived or natural chemical compounds with the potential to inhibit HDACs either specifically or non-specifically (pan HDACi), resulting in effective increases in histone acetylation and protein acetylation in general. Various compounds from different chemical classes are available for human use and are under investigation in Phase I to Phase III trials. On the basis of their chemical structure, HDACi have been broadly categorized into four main structural classes: carboxylic acids or short-chain fatty acids (SCFAs), hydroxamic acids, benzamides, and cyclic tetrapeptides.

Short-chain fatty acids (COOH group)

Chemically, these drugs are carboxylic acids or their derivatives with relatively weak HDAC inhibitory potential (millimolar concentration range). These drugs are mostly non-specific and exhibit relatively low bioavailability. These compounds bind to the active site of classical HDAC enzymes. In general, they work through Class I and Class II HDACs; specifically, sodium butyrate inhibits Class I and Class II HDACs (except HDAC6), and valproate inhibits Class I and Class IIa HDACs. These drugs readily cross the blood–brain barrier (BBB), and some of them (e.g., valproate) have been in use for many years to treat various neuropsychiatric disorders (as mood stabilizers, anticonvulsants, or antiepileptics). SCFA appear to have pleiotropic effects on other enzymes, as well. Once the molecular mechanisms become clear, novel SCFAs with good efficacy may emerge to be used alone or in combination with other drugs in the treatment of various leukemias, other non-cancer disorders, and neuropsychiatric diseases:



VPA

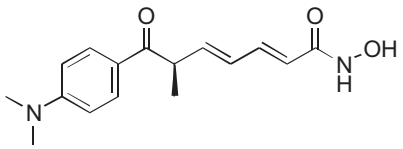


Butyric acid

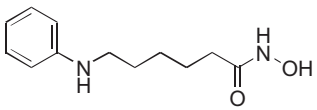
Hydroxamates (NHOH group)

Chemically, this class of compounds belongs to the hydroxyamides and their derivatives, which act as potent HDACi in the micromolar or even lower (nanomolar) range. They also exhibit inhibitory activity at all classical HDACs. The hydroxamate moiety appears to bind the zinc ion at the HDAC active site to inhibit the enzyme activity of most of the zinc-dependent HDACs including HDAC6. Members of this class, such as TSA and SAHA, are permeable to the BBB and show remarkable effects in preclinical neurological and psychiatric disease models. SAHA (vorinostat, zolinza) and romidepsin

received FDA approval in 2006 and 2010, respectively, for the treatment of persistent cutaneous T-cell lymphoma (CTCL). Researchers have now discovered a vast array of hydroxamates, with a linear cap or a bulky cap, some with no linkers, and some with branched chain moieties:



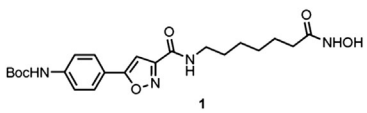
Trichostatin-A



SAHA

Linear cap hydroxamates

Some recent examples [91,92] of linear cap hydroxamate inhibitors include the following [93–97]:



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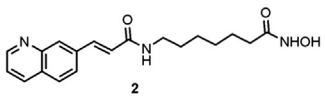

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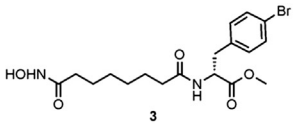

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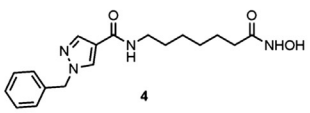

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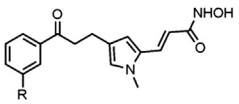
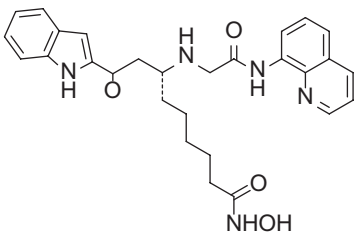


5, R=Cl
6, R=F

Branched hydroxamates

Examples include the bidirectional inhibitors 7, 8, and 9. Different branched chains in 7 and 8 target different residues in the enzyme active site, and data on 9 suggest a deep pocket occupancy [97–99]:



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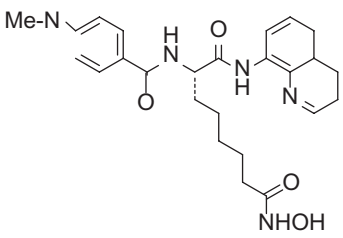

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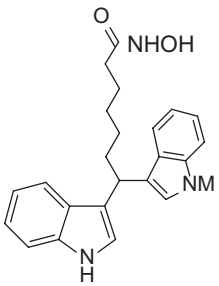
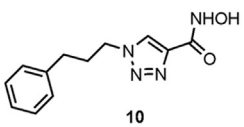


9

Hydroxamates with variable linker/linkerless regions

Inhibitors 10, 11, and 12 have variable linker regions. For example, 10 is a triazole, whereas inhibitors 13–16 are very rigid and sometimes are without a linker, as in 15 and 16 [100–104]:



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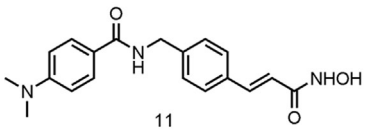

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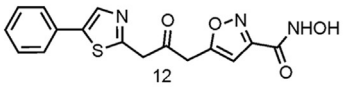

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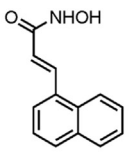

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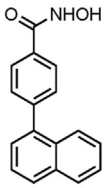

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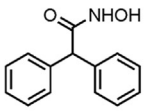

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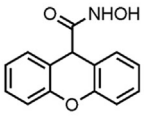
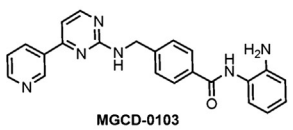


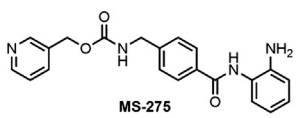
16

Benzamides ($PhNH_2$)

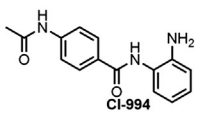
These HDACi are amide derivatives of benzoic acid, two of which are in the clinical trial phase. CI-994 (*N*-acetyldinaline) is a potent drug with antitumor activity at micromolar concentrations when given orally. CI-994 is used in combination with paclitaxel or carboplatin and has produced antitumor responses in several Phase I studies. MS-275 is a benzamide with radio-sensitizing properties on human prostate carcinoma and glioma cell lines. MS-275 is also in Phase II clinical trials for the treatment of various tumor types. It preferentially works to inhibit HDAC1, compared with HDAC2, 3, and 9. Similarly, MGCD-0103 also acts preferentially on Class I enzymes over Class II enzymes, with a 40-fold selectivity for HDAC1 and 3 over HDAC6 and 8 (HDAC1 $IC_{50} = 82$ n*M*) [105,106]:



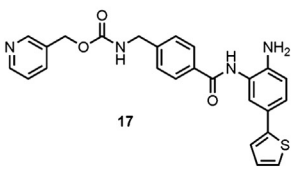
MGCD-0103



MS-275



CI-994



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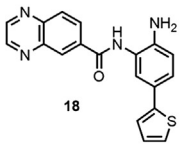

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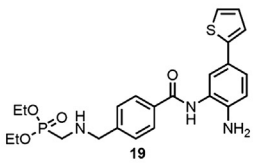


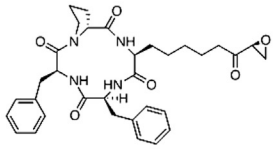
19

Cyclic tetrapeptides

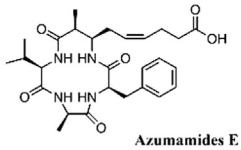
Cyclic tetrapeptides are a distinct class of drugs containing an α-epoxyketone group with the potential to alkylate the HDAC active site, thus deactivating the HDAC catalytic pocket. The HDAC inhibitory activity

of these compounds is a function of hydration of the electrophilic ketone group, which chelates zinc ions. Chemically derived substituted epoxyketone moieties such as pentafluoroethyl and trifluoromethyl ketones, sulfydryls, or retrohydroxamate show much higher efficacy. Some analogs of this group that have been piped into Phase I and II clinical trials include depsipeptide and aplidine, respectively. Some fungi-derived members of this class, such as the trapoxins A/B, are potent inhibitors and cause irreversible HDAC inhibition even at nanomolar concentrations by binding covalently to the HDAC via the epoxide group.

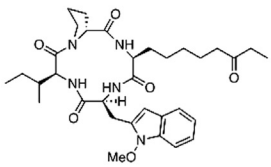
The cyclic hydroxamic acid-containing peptides (CHAPs) that reversibly inhibit HDACs at low-nanomolar concentrations are in fact hybrids of trichostatin and trapoxin. The compounds contain a hydroxamate side chain attached to the cyclic peptide core in place of the epoxyketone, and changes in the methylene chain length can lead to the development of many different CHAPs, each with its own properties:



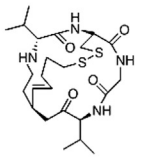
Trapoxin B



Azumamides E



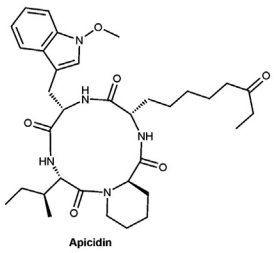
Apicidin



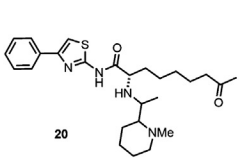
Romidepsin (FK-228, depsipeptide)

Ketone group

Apicidin is the most common example with a bulky cap group. Compounds 22 to 25 with trifluoroketone groups are also well studied [107–109]:



Apicidin



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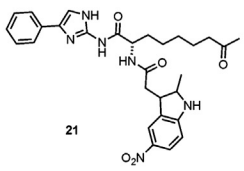

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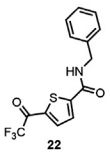

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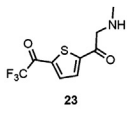

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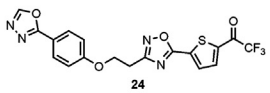

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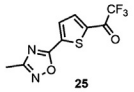

25

Constraints in the development of HDACi as therapeutics for CNS disorders

Most of the members of these HDACi classes are isoform non-selective, and this lack of target specificity is a serious concern in the long-term use of these drugs. Because HDACi show pleiotropic effects on multiple HDAC members, in addition to their effects on DNA demethylation of genes that have become repressed or silenced due to hypermethylation, these drugs may also induce global demethylation, which can have dangerous consequences, as a number of oncogenes, as well as retrotransposons, may become de-repressed or activated. The effects on DNA demethylation can be devastating if any of the oncogenes become activated.

Recent efforts to develop selective HDACi

Until now, only a few recently synthesized HDACi have some isoform specific selectivity [91,92,110]. These HDACi are derived synthetically as well as from natural sources. A synthetic benzamide derivative, MS-275 (now known as SNDX MS-275; Syndax Pharmaceutical, Inc.), has been shown to block the activity of only Class I HDACs (HDAC1, 2, and 3). It also has more specific activity toward HDAC1 compared with HDAC2 and 3 [111]. SNDX-275 infusion in NAc for a few weeks has been shown to reverse depression-like symptoms due to its transcriptional de-repressing effects on hundreds of genes in the mouse NAc, thus acting as a potential antidepressant [79]. Because SNDX-275 crosses the BBB easily, it can be administered orally with minimum adverse effects. Another benzamide derivative, MGCD-0103 (Methylgene Inc.), also seems to be isoform selective, as it has been shown to inhibit Class I and IV HDAC, without affecting Class II HDAC members [112], in its action in alleviating neuropathic pain [113]. Another HDACi, apicidin, a cyclic tetrapeptide, also has some selectivity as it acts on HDAC2 and 3 in the low nanomolar range, sparing HDAC1 and other members of Class II HDACs. It has slight inhibitory effects on HDAC8, but only when used in the higher nanomolar concentration range. Romidepsin (FK-228), another cyclic tetrapeptide, is very potent on HDAC1 and 2. Tubacin is another example of an HDACi which is highly selective for HDAC6 and known for the deacetylation of a microtubule component of α-tubulin [114].

Design of future HDACi for psychiatric disorders

Currently, there are two broad types of HDAC inhibitors: valproic acid and butyric acid. They are commonly used by researchers to gain an understanding of the mechanisms of HDAC inhibition in models of psychiatric disorders. These two inhibitors usually work at higher doses, because of their broad targeting action and also due to limited entry into the brain. Because of the limited knowledge regarding HDAC inhibitory mechanisms in alleviating psychiatric disorders, the role of individual HDACs in these disorders is not well understood. A better appreciation of the individual role of each HDAC could help revolutionize the field of HDAC inhibitor therapy in this context.

To date, efforts to design specific HDAC inhibitors have been limited due to the difficulties in purifying and expressing the enzymes. These enzymes are available commercially but at high cost, which limits their use. Further, not all of the enzymes process the same substrate, which is another

challenge faced by drug discovery research groups. Recently, a few research groups have tried to discuss the use of specific substrates for the individual class of HDAC enzymes [91]. Furthermore, lack of the inhibition data for any inhibitor against all of the HDAC members is not widely reported in the literature, suggesting that further profiling is necessary [92]. The only HDACi that have been tested against all members of the HDAC family are shown in Table 25.4.

A few of the most common HDACi in this table are in advanced stages of clinical trials, even with incomplete HDAC inhibitory profiling data [92]. Creating new and selective HDACi requires serious effort, but with the number of HDAC crystal structures (HDAC2, HDAC3, HDAC4, HDAC7, and HDAC8) now available, researchers should be able to reach certain conclusions for targeting the inhibition of specific HDAC enzymes. For example, one group has determined the specificity of HDAC6 inhibitors [91] against most of the HDAC enzymes, while another has validated HDAC6 inhibition against all 11 HDAC enzymes [93]. More focus should be on the design of HDACi by taking advantage of subtle differences in the active sites of each class of enzymes, going through minute details of the crystal structures.

The non-specificity of the classical HDACs is a main limitation in using HDACi as therapeutics, as they often elicit many adverse effects. This demands a shift toward the development of specific HDACi that can also be used as research tools to help us understand the role of individual HDACs in various brain disorders and their region-specific involvement. Until time-specific HDACi are developed, it will be prudent to adopt an alternative strategy, making analogs of VPA, an HDACi that has been in clinical use for the longest time. Some of the analogs of VPA may turn out to be more specific with better efficacy. If that is the case, they should be effective in the micromolar range, rather than the present milimolar range. It is somewhat surprising why chemists and researchers have not focused much on the short-chain fatty acid class of HDACi knowing that VPA has been shown to be effective in treating mood disorders.

A few CNS drugs, such as valproate (a mood stabilizer) and fluoxetine and imipramine (commonly used antidepressants), act through epigenetic mechanisms by altering histone acetylation/deacetylation. A highly prescribed tricyclic antidepressant, imipramine, restores hippocampal activity and mood in depressed mice through an increase in H3 acetylation at BDNF promoters III and IV, and the downregulation of HDAC5 [42]. Further, chromatin immunoprecipitation coupled with promoter microarray (ChIP-chip) experiments show that imipramine restores the mood in defeat-induced depressed mice by reversing the transcriptionally repressive H3 methylation marks (H3K9, K27) on many dysregulated gene promoters [89]. Fluoxetine, a commonly prescribed antidepressant that works as a selective serotonin reuptake inhibitor (SSRI), has been shown to reverse methyl mercury-induced depression-like conditions by increasing the acetylation of H3 at BDNF IV promoter. Recently, it was reported that an intradermal fluoxetine implant and intra-accumbens infusion of MS-275 successfully restored mood in this same social defeat model of depression by reversing gene expression patterns in the NAc. Interestingly, many of the genes restored by MS-275, here acting as an antidepressant, were common in fluoxetine-treated mice [79]. That a few psychiatric drugs in addition to valproate may also act as HDACi elicits optimism among those interested in treating mental disorders in a more effective way. However, whether the antidepressant activity of imipramine and fluoxetine is through an HDACi activity or a non-HDAC-dependent, cell-signaling-induced hyperacetylation of histone H3 is not yet clear. This prompts us to check prevailing neuroactive drugs for their role in epigenetic-mediated mechanisms. Until that time, we have selective HDACi for only a few drugs used to treat mental disorders. Additional drugs should be profiled or tested for HDACi

Table 25.4 IC_{50} of Common HDACi Against All HDACs (in nM)

Compound	HDAC1	HDAC2	HDAC3	HDAC4	HDAC5	HDAC6	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11
TSA	2	2	4	6	—	—	5	456	6	—	—
SAHA	68	3	48	101	—	90	104	1524	107	—	—
MS-275	181	164	2311	>10,000	—	>10,000	>10,000	>10,000	507±37	—	—
Romidepsin	36	1155	—	450	—	—	—	—	—	—	—
Apicidine	44	47	43	>10,000	—	>10,000	>10,000	575	>10,000	—	—
Trapoxin B	0.11	120	—	0.3	—	330	—	—	—	—	—
Azumanide-E	50	100	80	>50000	>10,000	12,000	9700	3700	28,000	—	—
CRA-A	—	—	—	—	—	—	—	—	—	—	—
CI-994	410	—	—	—	—	>10,000	>10,000	>10,000	—	—	—
MGCD-0103	34	34	998	>10,000	—	>10,000	>10,000	>10,000	ND	—	—
Tubacin	1400/995	6270	1270	17,300	3350	4/142	9700	1270	4370	3710	3790

activity. If these turn out to have partial or weak HDACi activity, efforts should be directed toward making analogs to potentially increase efficacy. If a few clinically available analogs turn out to be HDAC specific, it would increase their potential for testing on new conditions.

Apart from this, existing HDACi, although non-specific, can be paired with existing drugs for treatment during relapse in many psychiatric disorders as this might increase the efficacy of drugs. This was shown recently in a study where the combined treatment of fluoxetine and sodium butyrate showed enhanced antidepressant activity over the individual compound/drug alone in a mouse model. In this way, HDACi might act as "genome softeners" [115] that might help remove compact heterochromatin-like structures around repressed or silent genes and increase genome plasticity to reinvigorate a response to the existing psychiatric medications in refractory neuropsychiatric diseases. Thus, it is possible that in treatment-resistant depression, use of an HDAC inhibitor together with a current antidepressant drug may allow for a better outcome.

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Glossary

Histone acetyltransferase An enzyme that catalyzes the acetylation of histones.
Histone deacetylase An enzyme that catalyzes the deacetylation of histones.
Histone deacetylase inhibitor A drug that inhibits the enzyme histone deacetylase.

CHAPTER

The Social Environment and Epigenetics in Psychiatry

26

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CHAPTER OUTLINE

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KEY CONCEPTS

- Phenotypic variations may be caused by differences in long-term programming of gene function rather than variation in gene sequences *per se*, and studies of the basis for inter-individual phenotypic diversity should consider epigenetic variations in addition to genetic sequence polymorphisms.
- DNA methylation and chromatin structure are found in a dynamic balance throughout life that is maintained and defined by sequence-specific factors that deliver DNA methylation and histone modification to genes.

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- The fact that histone modifications and DNA methylation are potentially reversible processes suggests that psychosocial, pharmacological and social interventions may be used to treat mental illness.
- Epigenetic programming in the brain of rodents by maternal care early in life is a highly stable yet reversible process that results in long-term changes in gene expression.
- In our studies of social adversity in humans in early life, we found that aberrant DNA methylation of the glucocorticoid receptor promoter leads to decreased transcription of the gene and that this effect was associated with a history of early childhood abuse or neglect in humans.
- Microarray studies of gene expression programming by maternal care in rodents and childhood abuse or neglect in humans indicate that the epigenetic response to early-life experiences involves coding and non-coding regions of the genome and a number of genes related to stress and neuroplasticity.
- Several studies in rodents and humans indicate that genes related to neuroplasticity and stress are epigenetically labile in response to social factors in adulthood, outside of sensitive periods of development.
- Novel high-throughput technologies for epigenetic analysis that have enabled epigenetic studies of human populations and epigenetic changes throughout the genome must be matched by equally powerful analyses of phenotypes for an understanding of epigenetic contributions to behavior.

ABBREVIATIONS

5-mC 5-Methylcytosine
BIS-seq Bisulfite sequencing
CRF Corticotropin-releasing factor
DNMT DNA methyltransferase
GAD67 Glutamate decarboxylase 67
GC Glucocorticoid
GR Glucocorticoid receptor
HATs Histone acetyltransferases
HDAC Histone deacetylase
HDACi HDAC inhibitor
HPA Hypothalamic–pituitary–adrenal
LTP Long-term potentiation
MeDIP-seq Methylated DNA immunoprecipitation sequencing
miRNA microRNA
NGFI-A Nerve growth factor inducible A
PBMC Peripheral blood mononuclear cell
SAM *S*-adenosylmethionine
SES Socioeconomic status
TSA Trichostatin A
TSST Trier Social Stress Test

Introduction: social factors, genes, and gene expression programming

Social factors, particularly those that are encountered early in life such as parental care, can have profound effects on neurobiological trajectories and long-term consequences for mental health. It has been largely accepted that normal brain development depends upon a complex interplay between genetic and environmental factors. The field of behavioral epigenetics has provided a paradigm in which to examine novel mechanisms by which experiences can become "biologically embedded," influencing the development and maintenance of behavioral adaptations to environmental challenges.

It was once thought that patterns of gene expression are programmed such that, in the absence of pathology, they are largely resistant to variations in the environment during cellular development and differentiation. However, accumulating evidence now indicates that many different cell types execute distinct patterns of gene expression that are highly responsive to physiological and environmental cues

during development. To understand the relationship between environmental variation and variation in gene expression, a number of experimental rodent models have been developed to assess the influence of duration and timing of environmental exposures on behavioral outcomes. In addition, recent technological advances have enabled studies in human populations on unprecedented scales, enabling subphenotyping of large cohorts and the use of endophenotypes in human populations in conjunction with molecular assessments related to gene function, albeit typically in peripheral tissues. This research has provided evidence that a key component of the influence of environmental factors on gene expression programming lies in molecular mechanisms that operate "above the genome," termed epigenetic.

In this chapter, we will discuss several examples of approaches to rodent models and human cohort studies that have been applied to examine the influence of social factors on behavioral and mental health outcomes. We will highlight discoveries in the field that relate to epigenetic mechanisms that mediate risk and resilience to environmental challenges, with a particular focus on neurodevelopment. First, we will review basic epigenetic mechanisms and their known role in gene regulation. Next, we will discuss how epigenetic differences contribute to inter-individual variation in behavior. We will then highlight studies in animals and humans of the role of social factors in early life in mediating inter-individual differences in behavior via epigenetic mechanisms. We will complement this discussion with examples of these linkages outside of sensitive periods. Finally, we will discuss critical issues in the field for future studies of the role of the social environment and epigenetics in psychiatry.

How mechanisms of gene regulation "above the genome" contribute to inter-individual differences in behavior

Cell-type-specific patterns of epigenetic modifications are not exclusively genetically determined and are to some degree responsive to environmental signaling throughout life. The dynamic nature of epigenetic signaling contrasts with the static nature of the genetic code and provides a mechanism of gene-by-environment interactions that bridge inherited variation with variation originating from environmental and stochastic sources. It should be noted, however, that these sources of variation are not necessarily mutually exclusive, as genetic variation can interact with epigenetic variation [1]. However, in contrast to the relatively static nature of genetic variation, epigenetic variation is potentially amenable to psychosocial or therapeutic intervention. Thus, we and others have argued that a complete understanding of the origins of health and human disease—including psychiatric disorders—requires an integrative understanding of genetic, environmental, and stochastic contributions to epigenetic signaling [2,3]. As we will discuss below, with specific examples, there is accumulating evidence indicating that variation in neuroplasticity and behavior observed in humans and animal models as a function of social factors is associated with changes in gene function via epigenetic mechanisms.

Overview of epigenetic mechanisms

The epigenome consists of the chromatin and its modifications, as well as a covalent modification by methylation of cytosine rings found at the dinucleotide sequence CG [4], although recent evidence has indicated that 5-methylcytosine (5-mC) also occurs in non-CpG contexts in differentiated somatic cells [5,6]. The epigenome determines accessibility of the transcription machinery to DNA. Here, it is

necessary to distinguish between the open and closed configuration of chromatin. Densely packaged chromatin can be visualized microscopically and is termed heterochromatin, whereas open accessible chromatin is termed euchromatin [7–11]. Within heterochromatic regions, genes are inaccessible to the transcriptional machinery and therefore silent, whereas genes within euchromatic regions may be transcribed. Specific modifications to histones create a "histone code" that regulates accessibility of the transcriptional machinery. Another level of epigenetic regulation occurs via the activity of non-coding microRNAs (miRNAs) that regulate gene expression at different levels: silencing of chromatin, degrading mRNA, and inhibiting translation [12]. More details of epigenetic mechanisms of regulation of gene expression are given in Chapter 2 of this book.

Targeting of epigenetic modifications to specific genes

To date, knowledge about how specific environmental factors target select gene sequences remains poor, although we will review one such example below for maternal care. This gap in knowledge remains a significant challenge for the field. Transcription factors and repressors are known to recruit non-specific histone-modifying enzymes to specific genomic loci and target specific genes by recognizing specific *cis*-acting sequences in genes, binding to these sequences and attracting specific chromatin modifying enzymes to genes through protein–protein interactions [13].

A central question regarding gene-specific changes in DNA methylation associated with the environment concerns the targeting of these sequence-specific changes to specific loci in the genome. Because processes mediating DNA methylation and demethylation are non-specific, targeting must be achieved via other mechanisms. There is evidence that chromatin configuration can regulate the accessibility of genes to the DNA methylation machinery. For example, the histone deacetylase inhibitor (HDACi) trichostatin A (TSA), which leads to hyperacetylated chromatin, also leads to active DNA demethylation [14]. A change in histone acetylation is normally caused by transcription factors that recruit histone acetyltransferases (HATs), which may cause histone acetylation and facilitate demethylation. Examples of histone-modifying enzymes shown to interact with DNA methyltransferase 1 (DNMT1) are histone deacetylase 1 (HDAC1), HDAC2, the histone methyltransferases SUV3–9, and EZH2, a member of the multiprotein polycomb complex PRC2 that methylates H3 histone at the K27 residue [15–18]. DNMT3a was also shown to interact with EZH2, which targets the DNA methylation–histone modification multiprotein complexes to specific sequences in DNA [18].

Epigenetic mechanisms and mental health

In the broadest definition, epigenetic mechanisms refer to the combination of mechanisms leading to the long-term programming of gene function without a change in gene sequence. Epigenetic programming occurs primarily during development to generate the complex patterns of gene expression characteristic of complex organisms such as humans. Like gene expression, epigenetic mechanisms are somewhat dynamic in response to environmental exposures, especially though not exclusively during fetal development and early in life. Thus, particular phenotypic variation observed in human populations could be a function of epigenetic differences leading to the long-term programming of gene function as well as the genetic sequence. We and others have argued for this reason that the analysis

of inter-individual phenotypic diversity should take into account epigenetic variations in addition to genetic sequence differences [19]. We and others have also proposed that DNA modifications are maintained in an equilibrium between methylation and demethylation that is maintained as long as this equilibrium of sequence-specific factors engaging particular gene sequences is maintained [3,19]. This process is essential for normal development and the process of tissue-specific cellular differentiation. Physiological or environmental signals, which alter the signaling pathways in the cell, may result in altering this balance by activating or suppressing specific *trans*-acting factors.

Some critical environmental exposure, such as variations in maternal behavior, could alter the progression of epigenetic programming during development postnatally as well as *in utero*. Thus, variation in environmental exposures during these sensitive periods could result in epigenetic and therefore phenotypic differences later in life. Recent data suggest that social exposures early in life also impact the epigenome, resulting in differences in epigenetic programming and as a consequence in behavioral differences later in life [3]. As a result, certain early in life exposures that alter epigenetic programming may lead to later-life psychiatric disorders [3].

It is important to understand the mechanisms driving variations in epigenetic programming in order to identify the behavioral pathologies that result from such mechanisms. Unlike genetic mechanisms, the dynamic nature of epigenetic mechanisms implies that they are potentially reversible and amenable to therapeutic intervention [20]. Because various drugs used in the treatment of psychiatric disorders such as schizophrenia and mood disorders have known epigenetic effects, interventions targeting the epigenetic machinery could have important consequences for normal cognitive function. Once the rules governing the effects of environmental exposures on epigenetic processes are understood, it might be possible to design strategies to prevent and reverse deleterious environmentally driven epigenetic alterations.

The response of epigenetic machinery to environmental signals

Whether or not the DNA methylation at particular genomic loci is reversible has important implications for the possibility that the regulation of these loci by DNA methylation will be responsive to physiological and environmental signals throughout life. Enzymes that mediate changes in DNA methylation, such as DNMTs, are present in neurons [21], and there are data suggesting that DNMT levels in neurons change in certain pathological conditions such as schizophrenia [22]. The presence of DNMT in neurons suggests that the DNA methylation machinery is poised to act even in postmitotic cells, indicating that the DNA methylation is a balance of methylation and demethylation reactions [19]. miRNAs have been linked to behavioral pathologies in humans and regulate gene function through a variety of mechanisms, as has been extensively reviewed elsewhere [23–26]. These mechanisms are currently the subject of intense investigation in animal models as well. For example, altered miRNA expression in adulthood has been linked to early-life stress in rats [27], suggesting that it is responsive to environmental alterations in early life. Environmental or physiological events that interfere with signaling pathways may result in chromatin alterations [28]. Because signal transduction pathways are activated by cell-surface receptors, they may potentially serve as conduits for epigenetic change, linking the environmental trigger at cell-surface receptors with gene-specific chromatin alterations and reprogramming of gene activity [28]. Below, we will discuss an example of such a pathway that leads from maternal behavior to long-term programming of gene expression in the hippocampus.

Social factors associated with epigenetic modifications during sensitive periods of development

The maternal environment exerts a profound mediating role between environmental exposures and the developing offspring. In mammals, this mediation may occur as a result of a direct fetal exposure via the maternal–fetal interface, alterations in maternal physiology pre- and postnatally, or changes in mother–offspring interactions during early postnatal life [3]. A prominent and well-studied feature of maternal effects on neurodevelopment is its influence on the hypothalamic–pituitary–adrenal (HPA) axis, a major regulator of the endocrine response to environmental challenges. The regulation of circulating glucocorticoids (GCs) maintains homeostatic energy balance across the circadian cycle and mediates the physiological and behavioral response to stress. Output from the stress axis begins with sensory input on environmental variation that initiates a cascade of endocrine responses from the hypothalamus, culminating with the release of GCs in the form of cortisol or corticosterone that feed back on a variety of neural circuitry.

Ecologists have long recognized that chronic stressors (e.g., predation risk, resource availability, social interactions) play key organizing roles in ecosystems. Indeed, the mechanistic functioning of the HPA axis is highly conserved across vertebrate taxa, underscoring the biological importance of optimal glucocorticoid regulation [29–31]. It is increasingly understood that maternal stressors can induce preparative and adaptive programming in offspring via exposure to maternal GCs. Indeed, a diverse array of stressors can induce relatively permanent changes in the HPA axis of offspring via exposure to maternal stress during pre- and postnatal development: environmental effects on maternal state, predation pressure, quality of the rearing environment, and the unpredictability of the social environment [32]. The relative permanence of such changes in an ecological (natural world) context suggests that the effects of stress on the HPA do not reflect pathology but instead indicate adaptive responses that prepare offspring for environments where stressors are likely to be encountered [32,33].

A large number of human and animal studies have been designed to understand the role of early-life experiences in life-long psychopathology [3]. Human epidemiological studies have indicated that early-life experiences have enduring consequences for health in adulthood—including mental health—as a consequence of establishing long-term health trajectories [34]. For example, early-life low socioeconomic status (SES), a measure of relative financial, educational, and social position, strongly predicts a wide range of mental health problems in adulthood, including schizophrenia and depression [3,35,36]. Likewise, early adverse experiences such as physical abuse or neglect are well-known risk factors for mental health problems later in life [37]. Children who experience parental neglect as a result of institutionalization in early life show profound intellectual impairment and failure to completely catch up, even with social intervention [38]. Childhood physical and sexual abuse also impairs intellectual function and increases the risk of affective disorders and suicide [39]. Environmental experiences during early life have been suggested to exert an enhanced impact on health trajectories in part because early postnatal development is a time of enhanced plasticity [40,41].

Elucidating the biological mechanisms underlying effects of early social experiences on later mental health is challenging in humans for reasons that include limited access to relevant biological material. More is known about the pathways altered by adversity than other forms of early social experience. Studies in animal models have suggested that early-life stress impairs neuroplasticity in brain regions such as the hippocampus and has a lasting impact on endocrine systems underlying the response to psychosocial stressors [35,42]. Laboratory rodent models have been particularly useful in identifying mechanisms of epigenetic regulation in the brain that have then been used to generate hypotheses in humans.

Biomedical studies of humans and laboratory animals indicate a profound effect of parental care early in life on the epigenetic programming of the stress axis and associated behaviors.

Maternal care, epigenetics, and the HPA axis: laboratory animal studies

Research findings by Weaver, Meaney, Szyf, and colleagues in the early 2000s launched epigenetics to the forefront of research on mechanisms leading from maternal behavior to long-term programming of gene expression in the offspring. Earlier work by the Meaney laboratory and others had established that naturally occurring differences in maternal care in the early postnatal period—during the first week of life in the rat—lead to long-term effects on stress and stress-related behavior. The offspring of mothers who naturally exhibit high levels of care show elevated transcript abundance of the glucocorticoid receptor in the hippocampus, enhanced negative feedback sensitivity, and a more modest response to stressors in adulthood [43]. Cross-fostering studies showed that this phenotype is directly attributable to maternal behavior rather than factors related to the prenatal environment, as offspring phenotype was shown to match that of the adoptive mother rather than that of the biological mother [44]. Weaver and colleagues [45] showed that the accompanying change in glucocorticoid receptor (GR) expression was regulated by DNA methylation of the $GR1_7$ splice variant in the hippocampus by inhibition of the binding of NGFI-A, a transcription factor that drives GR expression. $GR1_7$ is one of at least 11 untranslated first exons of the GR gene. Though it is ubiquitously expressed in virtually all cells, levels of expression of GR vary and are controlled in part by tissue-specific expression of GR exon 1 splice variants (this is also true for the human GR exon 1, as will be discussed) [46,47]. In the hippocampus, $GR1_7$ was previously shown to vary in expression as a function of the level of maternal care received [47]. Interestingly, relatively high levels of DNA methylation were maintained among the offspring of low-maternal-care mothers, whereas offspring of high-maternal-care mothers showed demethylation of this promoter during the first week of life, coinciding with emergence of differences in maternal care between the two litter types. The results implied that DNA demethylation (through a yet unknown process) leads to an increased number of GRs and an attenuated response to stress. DNA methylation differences were stable throughout adulthood in these animals, but were reversible by infusion of TSA, a histone deacetylase inhibitor, which also led to increased gene expression in hundreds of other genes [48]. Likewise, lower levels of DNA methylation observed among the offspring of high-maternal-care mothers resembled those of offspring of high-maternal-care mothers given central infusions of the methyl donor *S*-adenosylmethionine (SAM), indicating that enzymes responsible for DNA methylation were poised to act in the adult brain in response to a methyl donor.

A recent study has challenged the idea that $GR1_7$ transcript is regulated by DNA methylation of the NGFI-A response element in rats exposed to stress paradigms that lead to altered NGFI-A levels, although stress does appear to modulate the methylation of other CG sites within the $GR1_7$ promoter [49]. It is likely that DNA methylation of $GR1_7$ gene expression involves the binding of additional transcription factors and/or is context and brain-region specific. It is also likely that the $GR1_7$ is itself part of a response mechanism that involves additional splice variants of GR and perhaps other transcription factors.

We performed a microarray analysis of DNA methylation, H3K9 acetylation, and gene expression in a 7 million base pair region containing the GR gene in the rat hippocampus [50]. We found that epigenetic differences in adulthood that were associated with early maternal care occurred in clustered regions of up to 100 kb but were nonetheless exquisitely patterned, whereby increased transcription

occurred in conjunction with hyperacetylation and hypermethylation of exons and hypomethylation of promoters. We found epigenetic differences in association with altered transcription as a function of maternal care across several GR_1 splice variants. Large epigenetic differences were noted in proximity to the transcription start site of GR, within the first coding exon (exon 2) and within GR introns, suggesting there may be additional regulation of GR via yet-to-be-identified non-coding RNAs within the GR locus. These data were the first to link epigenetic changes across both coding and non-coding regions in the mammalian brain, and they implicate a non-random epigenetic programming across large-scale loci in response to differences in early care. Accumulating evidence indicates that additional genes in neural pathways mediating the stress response are epigenetically regulated by DNA methylation of gene regulatory elements in association with early-life stress—for example, arginine vasopressin in the hypothalamus [51], BDNF in the hippocampus [52], and GAD67 in the prefrontal cortex [53].

These postnatal programming effects appear to derive from environmentally induced alterations of maternal–neonatal interactions involving systems that determine methylation patterns of GR gene promoter sequences and additional loci. It will be important to understand the precise nature of the maternal–neonatal interaction that mediates these changes. For example, there is evidence that artificial stimulation of pups with a paintbrush as a substitute for maternal licking can alter DNA methylation of a promoter region of the estrogen receptor alpha gene in the preoptic area of the hypothalamus [54]. These data have important implications for studies of transgenerational impacts related to maternal care via epigenetic mechanisms, via *behavioral* mechanisms of inheritance rather than gametic inheritance, as maternal behavior is associated with levels of maternal care provided by offspring to their progeny [55].

Parental care, epigenetics, and the HPA axis: human studies

Recent advances in genomics have provided new means to address these questions in large numbers of human subjects in an increasingly comprehensive and powerful manner. In the previous section, we discussed evidence from our studies of widespread but specific epigenetic and transcriptional alterations of the GR gene extending far beyond the GR promoter associated with differences in maternal care [50–53]. Thus, there is mounting evidence that epigenetic mechanisms coordinate widespread changes in gene expression in response to differences in early maternal care or adversity.

Altered DNA methylation of the GR promoter has also been found to be associated with social behaviors and HPA dysfunction. In one of the earliest reports, DNA methylation of GR promoter in infants' cord blood was found to differ as a function of maternal mood during pregnancy and correlate with infants' cortisol response [56]. These data suggest that GR promoter methylation in the brain and in lymphocytes is under epigenetic control as a function of maternal physiology or perhaps differences in maternal care early in the life of the offspring. We examined postmortem brain tissue from adults with well-characterized life histories to investigate the influence of early-life adversity on glucocorticoid receptor DNA methylation in adults with a history of trauma. Our focus was on individuals with a history of severe physical or sexual abuse or neglect during childhood, which is common among suicide victims and is an important risk factor for suicide [3,37]. We examined the GR1F promoter in the hippocampus of human suicide victims and controls [57]. Family dysfunction and childhood adversity are linked to altered HPA stress responses and an increased risk for suicide. The promoter region we examined is upstream of one of several untranslated exon 1 splice variants that are known to regulate tissue-specific expression of GR, akin to the function that the GR exon 1 splice variants serve in the

rodent [46]. The study included three conditions: (1) suicide completers with a history of childhood abuse or severe neglect, (2) suicide completers without a history of childhood abuse or neglect, and (3) individuals who had neither committed suicide nor had a history of childhood abuse or neglect. A fourth group of non-suicide victims with a history of abuse or neglect was not available, partly due to the fact that tissues from such a "control" group are exceedingly rare and were unavailable for our study. In this study, we found that the GR gene was differentially methylated among suicide victims with a history of abuse in childhood, but not among suicide victims with a negative history of childhood abuse, compared with control individuals without a history of suicide. The data suggest that epigenetic processes might mediate the effects of the social environment during childhood on hippocampal gene expression and that stable epigenetic marks such as DNA methylation might then persist into adulthood and influence vulnerability for psychopathology through effects on intermediate levels of function, such as activity of the HPA axis that regulates the stress response. However, it is still unclear whether the epigenetic aberrations were present in the germ line, whether they were introduced during embryogenesis, or whether they were truly changes occurring during early childhood. We also do not yet know the extent to which parental factors *per se* play a role in this phenotype. Despite these important caveats, these data were the first to link the early-life environment to changes in the GR gene in humans. The data parallel those for the rodent study mentioned above, although in a very different context.

We have applied high-throughput approaches to examining DNA methylation, chromatin modifications, and mRNA expression in gene regulatory, coding, intragenic, and intergenic regions in humans in a study that paralleled that described above in rats. We analyzed the GR gene locus by interrogating a 7-MB region containing the GR gene in hippocampi of adult suicide victims who were abused early in life compared with controls using high-throughput DNA microarray [58]. The GR gene locus shows substantial conservation with the same locus in rodents, with an almost identical order or orthologous genes across the locus. Like the study in the rat [50], methylation levels were non-randomly distributed across the locus, indicating that stochastic processes are unlikely to account for the range of variation that we observed in this study. Proximal to the GR gene itself, we found a large region hypermethylated in suicide completers relative to controls within the first coding exon of the GR gene and its proximal promoters, extending previous observations of hypermethylation of the GR1F promoter among suicide victims with a history of abuse [57]. This analysis also revealed differences in DNA methylation in intragenic regions of the GR gene. At this time, we can only speculate that unrecognized non-coding RNAs may reside within this region and affect GR expression. Other differences were discovered within coding regions and the 3′-UTR of the GR gene. These data suggest that GR is epigenetically labile in response to the early-life social environment in both rodents and humans, although the specific alterations that we observed are not identical in both species [58]. Nevertheless, the data indicate that the animal model of parental care may have broad applicability for translational studies aimed at understanding the consequences of epigenetic modification of the GR in humans [3].

Social factors associated with epigenetic modifications outside of sensitive periods of development

Social stress, epigenetics, and the HPA axis: laboratory animal studies

One of the most common paradigms used to model genetic and epigenetic mechanisms in stress-related psychiatric disorders in laboratory animals is chronic social defeat. The paradigm consists of repeated

presentations of an aggressive individual and yields two types of behavioral outcomes: (1) stress-vulnerable individuals who avoid subsequent social contact and show other depressive-like behaviors, and (2) stress-resilient individuals. Interestingly, aspects of the behavioral outcomes associated with social defeat have been shown to be transmitted transgenerationally via the father's sperm [59]. When mice were subjected to social defeat and then bred to non-stressed females or when the defeated father's sperm was used for *in vitro* fertilization, offspring showed a decreased latency to become immobile in a forced swim test, a standard measure of depressive-like behavior. However, there was no reduction in social avoidance among offspring generated by *in vitro* fertilization, in contrast to those bred through standard mating, suggesting that aspects of the father's behavior during mating that may have influenced reproductive success or maternal behavior and physiology are necessary for the expression of avoidance behavior, at least in the experimental context that was examined.

Transgenerational impacts of social experiences have been studied using paradigms involving disrupting social hierarchies and environmental enrichment in laboratory mice. For example, when hierarchies are disrupted by repeatedly changing a rodent's cage mate, anxiety is increased in both mothers and female offspring [60]. Interestingly, disrupted social interaction and increased anxiety are also observed among second- and third-generation female offspring as a result of transmission via the paternal line [60]. Strategies whereby mice are exposed to an enriched environment, such as novel objects or exercise, have also shown that the enriched environment influenced anxiety and social behavior. In one study, adolescent females that were exposed to an enriched environment showed increased social interaction and enhanced learning and long-term potentiation (LTP) in offspring [61]. A recent study that has garnered considerable interest indicates that environmental information regarding fearful experiences may be transmitted transgenerationally in a manner that is independent of social transmission. The authors of this study showed that behavioral sensitivity to a novel odor (but not other odors) that was paired with shock in a fear-conditioning experiment could be transmitted to first- and second-generation offspring [62]. *In vitro* methods were used to demonstrate paternal transmission via the gametes, implicating an epigenetic mechanism. The authors also reported that a specific olfactory receptor (Olfr151), known to be sensitive to the odor used in the fear-conditioning experiment, showed differences in methylation as a function of conditioning in both the father and the offspring. Additional work is needed to understand how such a mechanism is transmitted via the gametes to direct epigenetic change at this specific olfactory receptor. Nevertheless, the results are exciting because they demonstrate the potential involvement of epigenetic processes in transmitting (or perhaps forecasting) information learned in one generation to additional generations.

Studies of specific genes that are altered as a function of social experience using a social defeat paradigm have documented altered hippocampal BDNF transcription with repeated encounters with an aggressive individual in stress-vulnerable individuals. These changes in gene expression occur together with epigenetic changes in the *BDNF* gene. Among defeated individuals, one study found lower transcript abundance of specific BDNF transcripts and increased levels of repressive histone marks on the *BDNF* gene with social defeat stress [63]. In a study of the contribution of individual differences to social defeat stress vulnerability, epigenetic changes that include increased histone acetylation and activation of BDNF VI transcripts were associated with a stress-resilient phenotype [64]. In the hippocampus, medial prefrontal cortex, and dorsal raphe nucleus, the acetylation of histones and the expression of histone-modifying enzymes are also correlated with behavioral outcomes associated with chronic social defeat stress [65]. Other groups have shown that genes associated with the regulation of the HPA axis are epigenetically modified by this form of social stress. Mice that spent less time in a

social interaction zone after social defeat display long-term demethylation of the corticotropin-releasing factor (CRF) gene in the hypothalamus. This change in methylation occurs in the context of overexpression of CRF, an overactive HPA axis, and behavior characteristic of social avoidance [66]. However, mice that are stress resilient do not show these epigenetic changes and spend more time in the social interaction zone after exposure to the same social defeat procedure.

Social stress, epigenetics, and the HPA axis: human studies

In contrast to the studies described above in rodents, to date fewer studies have examined the role of epigenetic modifications in association with the social environment in humans. Recent work has indicated that mildly stressful social experiences can lead to rapid epigenetic modifications in the human genome. A study using the Trier Social Stress Test (TSST) has found short-term increases in DNA methylation of the oxytocin receptor gene in peripheral blood [67]. DNA methylation status of the oxytocin receptor gene is also associated with individual variability in neural responses within brain regions supporting social perception [68]. In another study, the TSST showed gender-specific differences in methylation of the GR gene, with greater methylation of GR among females compared with males and a corresponding decrease in salivary cortisol during administration of the test [69]. Greater stress has also been found in association with lower methylation of the catechol-*O*-methyltransferase Val^{158} allele and more inefficient prefrontal activity [70].

Conclusion

A more complete understanding of the role of epigenetic mechanisms in perinatal programming will be afforded by studies that address several basic questions. First, in what contexts is the epigenome labile in response to early environment? Are there indeed critical time windows for the influence of the environment on epigenetic trajectories? A number of studies have linked early-life events to changes in neuroplasticity that have a lasting impact on endocrine systems mediating the response to stress [71]. It is not always clear, however, which cell types are relevant to the question under study. This is particularly problematic for studies in humans, where access to neural tissue is non-existent or limited. Peripheral cells such as peripheral blood mononuclear cells (PBMCs) offer an avenue to examine the HPA, as PBMCs are sensitive to endocrine modulation of HPA. Whole blood has also been used, but each tissue type is known to be sensitive to differences in constituent cell numbers, which may bias the results [72,73]. However, in studying environmental impacts prospectively in children, it is not often possible to obtain blood samples, and other tissues must be used. The most commonly used tissue in such epigenetic studies are buccal cells from mouth swabs or saliva. Intriguingly, there is some evidence that such tissue is responsive to early-life adversity, although perhaps not via epigenetic changes in GR *per se* [74]. Buccal cells complement studies of adversity in neurons in the sense that they do represent cells with a common embryonic origin. Such studies will provide a valuable means of resampling to examine epigenetic variance over time and with interventions. In animal studies, a goal going forward for translational work will be to identify labile epigenetic regions like the glucocorticoid receptor that can be assessed in brain and blood in order to generate hypotheses and biomarkers that can be examined in humans. Such research stands to offer critical insights into the manner by which the biological embedding occurs during the perinatal period.

Finally, identifying the specific conditions in which psychopathology arises as a function of early-life environment will shed light on the ultimate causes of epigenetic plasticity and the reasons for why some regions of the genome appear to respond to the environment in early life. For example, Barker's hypothesis, the proposal that pathological outcomes result from reduced fetal growth [75], stimulated research on a variety of health-related conditions arising from early environmental exposures [76]. This research revealed that nutrition and parental care can alter health trajectories in a manner consistent with that of an adaptive response, as both early undernutrition and overnutrition can lead to the same pathological outcomes (e.g., metabolic syndrome, cardiovascular disorders) [76,77]. Thus, the range of responses to early adversity suggests instead that pathology may arise as a function of mismatch between the early-life environment and the later-life environment rather than as a consequence of early-life dysfunction. This distinction is potentially important, because it implies that, for animal and human studies, specific environmental conditions may exist in which pathological responses may instead confer an apparently adaptive advantage.

In the studies we reviewed above from our group examining the epigenetic signature of maternal care in rodents and parental care in humans, we performed DNA microarray studies of the epigenome, which remain the most cost-effective means of targeting specific loci for epigenetic analyses. However, the resolution (~200 bp) of these approaches is less than sequencing-based analyses. As the cost of sequencing continues to decrease, it is now becoming feasible to employ sequencing-based single nucleotide resolution epigenetic analyses of DNA methylation (via MeDIP-seq or bisulfite sequencing [BIS-seq]) and chromatin modifications (via ChIP-seq) of the whole genome. These approaches have important limitations, as have been reviewed elsewhere [78]. Whole genome sequencing generates enormous datasets that require novel approaches for analysis. However, there are encouraging signs for a number of standard platforms that analytical methods are becoming standardized and within the reach of scientists without extensive bioinformatics training, including web-based analysis software such as Galaxy (galaxyproject.org). High-throughput studies such as the ones described above in the rat and human open up a number of questions—undoubtedly more than are answered. For data to be interpretable within this new paradigm, it is clear that these technological advances must be coupled with equally powerful phenotypic methods using appropriate cell-types and conditions.

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Glossary

Chromatin Histone proteins associated with the cell's DNA that regulate its accessibility to gene transcription machinery. Chromatin comes in two forms: heterochromatin, where the DNA is tightly coiled and therefore inaccessible to the transcriptional machinery, and euchromatin, where the DNA is more loosely associated with histone proteins.

Epigenome The overall epigenetic state of a cell that serves as an interface between the environment and the genome.

Histone code The specific pattern of histone protein modifications that delineate the parts of the genome to be expressed at a given point in time in a given cell type.

Phenotype Any observable characteristic of an organism, including its behavior. An organism's phenotype is a product of its genetics and its environment.

Psychopathology The manifestation of mental illness in the form of phenotype, including abnormal behavior and physiology.

CHAPTER

Nutritional Effects on Epigenetics in Psychiatry

27

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KEY CONCEPTS

- Nutritional factors in the diet can epigenetically influence the pathogenesis of psychiatric disorders. This influence can take place prenatally and postnatally.
- Nutritional agents that act epigenetically could be used in the management of psychiatric disorders.

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ABBREVIATIONS

AD Alzheimer's disease
C Cytosine
IGF2 Insulin-like growth factor 2 gene
LINE-1 Long interspersed nucleotide element 1
miRNA microRNA
MTHF Methyltetrahydrofolate
MTHFR Methylenetetrahydrofolate reductase
SAMe *S*-adenosylmethionine
Sat2 Satellite 2 DNA sequence
SNRI Selective noradrenaline reuptake inhibitor
SSRI Selective serotonin reuptake inhibitor
T Thymine

Introduction

There is increasing evidence that epigenetic mechanisms of gene expression play a major role in the pathogenesis of psychiatric disorders [1,2]. It is also clear that epigenetic mechanisms of gene expression can be influenced by the environment [3]. One such well-established environmental factor that is known to influence epigenetic mechanisms of gene expression is an individual's nutrition. An individual's nutrition is comprised of water, metabolic fuels (mainly carbohydrates and lipids), proteins, minerals, vitamins, and essential fatty acids [4]. This chapter discusses the role of nutrition on epigenetics in relation to psychiatric disorders.

Nutritional epigenetics: the link between nutrition and epigenetics

It is now well established that an individual's nutrition affects his or her epigenome, and epigenetics has been described as a bridge between an individual's nutrition and his or her health [5]. The study of the relationship between nutrition and epigenetics has given rise to a new discipline, nutritional epigenetics [6]. Nutritional epigenetics seeks to explain the effects of nutrition on gene expression. Thanks to the study of nutritional epigenetics, food, in addition to being chemically converted to energy and body matter, is now also known to be a conditioning environment that shapes the activity of the genome and the physiology of the body [6]. Nutrients and bioactive food components can modify epigenetic phenomena and alter the expression of genes at the transcriptional level [5]. Nutrition can influence epigenetic mechanisms in a number of ways [7]: through the availability of substrates used in epigenetic reactions; by direct effects on proteins involved in epigenetic marking; by direct effects on the genome; and by the selection and propagation of cells with particular epigenetic profiles. These effects can occur throughout the body [8]. They can occur prenatally [9], postnatally [9,10], and throughout the lifespan [9], and they can be transmitted from one generation to the next [11]. These effects have also been implicated in the development of disease [12]. A list of some of the nutrients that affect epigenetic mechanisms of gene expression is given in Box 27.1.

Effect of nutrition on DNA methylation

Nutrition can influence DNA methylation patterns in different tissues of the body, probably in a tissue-specific manner or transgenerationally; that is, nutrition can influence the subsequent progeny

BOX 27.1 SOME NUTRIENTS AFFECTING EPIGENETIC MECHANISMS OF GENE EXPRESSION

DNA Methylation

Folic acid (vitamin B_9), vitamin B_{12}, methionine, choline, high-fat diet, alcohol, green tea, selenium, genistein, betaine (trimethylglycine), vitamin B_2, vitamin B_6

Histone Modifications

Histone Acetylation

Butyrate, diallyl sulfide, curcumin

Histone Methylation

Folic acid, choline, methionine, vitamin B_6, vitamin B_{12}, riboflavin, zinc

Histone Biotylation

Biotin

Poly ADP-Ribosylation of Histones

Tryptophan, niacin

RNA-Mediated Regulation of Gene Expression

Methyl donors, folic acid, curcumin, genistein, retinoic acid

[12,13]. In animals and in *in vitro* laboratory models it has been shown that an excess or a deficiency of methyl donors (e.g., folate, methionine, choline), protein restriction, a high-fat diet, alcohol, green tea, selenium, and genistein, a phytoestrogen present in soy, can alter the DNA methylation patterns of several genes [13,14]. Compared with animal and *in vitro* laboratory studies, relatively few studies have investigated the influence of nutrition on the DNA methylation patterns of specific genes in humans [13]. However, in humans it has been shown that nutritional factors such as calorie restriction, folic acid, and vitamin B_{12} intake, as well as the consumption of soy isoflavones (a type of organic compound found in soy beans), can affect the DNA methylation status of a few genes [13].

Most work done so far on the effect of nutrition on DNA methylation has been on the hematopoietic drugs folic acid (vitamin B_9) and cyanocobalamin (vitamin B_{12}) during the fetal and early postnatal periods of life. These nutrients are necessary for the conversion of homocysteine to methionine. In order for this conversion to take place, folic acid is metabolized in the body to methyltetrahydrofolate (MTHF). MTHF acts as a methyl donor during the conversion of homocysteine to methionine, and in the process MTHF is converted to tetrahydrofolate. Methionine is metabolized to *S*-adenosylmethionine (SAMe) (Figure 27.1), the major methyl donor for most biological reactions in the body, including the methylation of DNA [15].

In addition to folic acid and vitamin B_{12}, other nutrients are needed for DNA methylation [16]. Choline, a derivative of phosphatidic acid, is an indirect methyl group donor for one-carbon metabolism, a metabolic pathway in the body centered around folic acid [17]. Betaine (trimethylglycine) can contribute to methionine homeostasis through the donation of a methyl group to homocysteine, resulting in the conversion of homocysteine to methionine. The water-soluble vitamins B_2, B_6, and B_{12} have important catalytic roles in one-carbon metabolism [17]. Vitamin B_6 serves as a coenzyme for a key step in folic acid metabolism. Vitamin B_2 serves as a precursor for a cofactor involved in folic acid metabolism. Vitamin B_{12} is the coenzyme of methionine synthase, the enzyme that catalyzes the conversion of homocysteine to methionine (Figure 27.1) [15]. Adequate amounts of dietary zinc are also required for one-carbon metabolism to function properly [16].

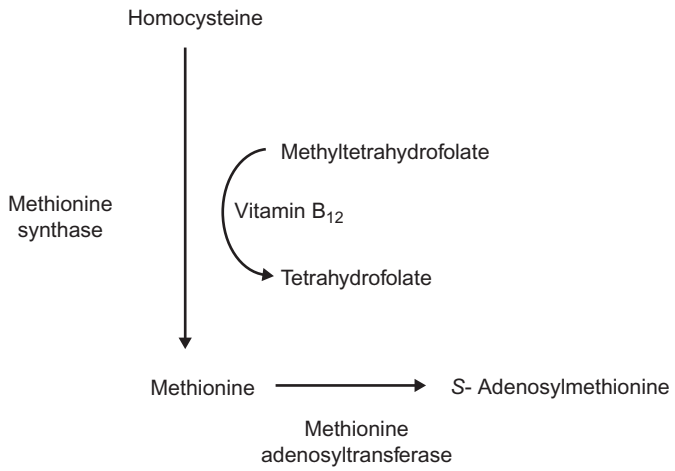

FIGURE 27.1

Schematic representation of conversion of homocysteine to methionine and of methionine to *S*-adenosylmethionine.

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Effect of nutrition on histone modifications

Nutrition can also influence various biochemical modifications of histones, especially histone acetylation [5,18,19]. Some nutrients, such as the short-chain fatty acid butyrate from resistant starch or fiber fermentation and diallyl sulfide from garlic, can inhibit histone deacetylase, thereby causing hyperacetylation of histones [18]. Curcumin, whose medicinal properties have long been recognized in India and Southeast Asia, can inhibit histone acetylation due to inhibition of the enzyme histone acetyltransferase [5]. Folic acid, choline, methionine, vitamin B_6, vitamin B_{12}, riboflavin, and zinc are all necessary for the supply of methyl groups by methyl donors for histone methylation to occur [16]. In the diet, other nutrients that are required in optimal amounts are biotin, which is a cofactor in fatty-acid synthesis by carboxylase enzymes and is required for normal histone biotinylation, and tryptophan and niacin, which supply NAD^+ for poly ADP-ribosylation of histones (NAD^+ is also required for class III histone deacetylases) [16]. Calorie restriction has also been shown to affect histone acetylation, histone deacetylation, and histone methylation [5].

Effect of nutrition on RNA-mediated regulation of gene expression

A deficient diet leading to methyl donor deficiency can cause alterations in the expression of microRNA (miRNA). This state of altered miRNA expression due to a methyl-donor-deficient diet may lead to disease states such as cancer and non-alcoholic steatohepatitis in rodents [5,20]. Experimental studies have shown that a deficiency of folic acid in the diet can lead to altered expression of miRNAs [5]. Bioactive food components such as curcumin, genistein, and retinoic acid have also been shown to alter the activity of miRNAs in experimental studies [5,20].

BOX 27.2 NUTRITIONAL EFFECTS ON EPIGENETICS IN PSYCHIATRY

Prenatal Effects

- Dutch Hunger Winter of 1944–45 resulted in a twofold increase in schizophrenia.
- The Great Chinese Famine of 1959–61 resulted in a twofold increase in schizophrenia.
- Prenatal undernutrition has been implicated in the pathogenesis of other psychiatric disorders; epigenetically mediated mechanisms have been implicated.

Postnatal Effects

- Nutrients involved in one-carbon metabolism have been linked to the occurrence of schizophrenia, major depressive disorder, and bipolar disorder.
- These effects can be influenced by polymorphisms in the gene encoding methylenetetrahydrofolate reductase (MTHFR).

Transgenerational Effects

- At present there is no experimental evidence for transgenerational effects of nutrition in psychiatric patients.

Nutritional effects on epigenetics in psychiatry

As far back as 1961, it was noted that when large oral doses of methionine were given to patients with schizophrenia some of the patients developed marked alterations in behavior [21]. This finding contributed to development of the methylation hypothesis of psychoses, according to which methylation of amines in the brain could underlie the pathogenesis of these disorders [22]. Later, it was suggested that the methylation hypothesis for the psychoses could involve methylation of DNA, rather than amines, in the brain [23,24]. Nutritional influences on epigenetics in psychiatry can take place during the prenatal (fetal) period, as well as after birth, during the postnatal period. Indeed, nutrition can influence epigenetics in psychiatry throughout the lifespan of an individual. Nutritional influences on epigenetics in psychiatry are summarized in Box 27.2.

Effect of prenatal nutrition on epigenetics in psychiatry

Both prenatal overnutrition and undernutrition can act epigenetically to influence the propensity to develop psychiatric disorders in the offspring [25]. However, most studies to date have studied the role of undernutrition on epigenetics in psychiatry. Schizophrenia is the psychiatric disorder that has been most extensively linked to prenatal undernutrition. Susser and Lin [26] were the first researchers to show that there is a link between severe nutritional deficiency and the offspring's risk of developing schizophrenia [27]. They examined the association between nutrition and schizophrenia using the unusual circumstances caused by the Dutch Hunger Winter of 1944–45. In October 1944, the German army stopped food supplies to the Western Netherlands as punishment for the involvement of the Dutch in the planned invasion of Europe by the Allies. Prior to the food supplies being stopped, food availability was adequate. In May 1945, after the retreat of the Germans, food became plentiful again. Between October 1944 and May 1945, the Dutch people, especially those in the larger cities, received increasingly smaller food rations, a situation that was aggravated by an unusually severe winter in 1944. Hence, Dutch women who were pregnant during this period experienced decreasing food supplies at varying periods of pregnancy. Analysis of rates of schizophrenia based on case data obtained

from the Dutch national psychiatry registry indicated that early severe prenatal nutritional deficiency resulted in a twofold increase in risk of schizophrenia in the offspring.

The findings of Susser and Lin [26] were supported by those of St. Clair et al. [28] who studied the association between severe prenatal nutritional deficiency and risk of schizophrenia in the offspring during the Great Chinese Famine of 1959–1961. These authors examined the rates of schizophrenia in those born before, during, and after the famine in one of the most famine-affected regions of China. Using sophisticated epidemiological methods, they found that the rates of schizophrenia were twofold higher among individuals born during the period of the famine. These findings on the same study population were replicated later by the same research group in another study [29]. Although other mechanisms cannot be ruled out, one very plausible mechanism by which prenatal famine increases the risk of schizophrenia in the offspring is by altering epigenetic mechanisms of gene expression [30,31]. Prenatal undernutrition has also been implicated in the pathogenesis of other psychiatric disorders such as major depression disorder and bipolar disorder [32], schizoid personality disorder [33], antisocial personality disorder [34], hyperactivity and peer problems during childhood [35], and cognitive impairment during late adulthood [36].

There is experimental evidence supporting the idea that epigenetic mechanisms of gene expression underlie the effects of prenatal famine on the susceptibility to develop psychiatric disorders. Heijmans et al. [37] showed that individuals who were exposed prenatally to the Dutch Hunger Winter of 1944–45 had, six decades later, less DNA methylation of the imprinted insulin-like growth factor 2 (*IGF2*) gene compared with unexposed individuals. Tobi et al. [38] later showed in individuals from the same study population that there are persistent and widespread changes in DNA methylation and that these changes are affected by the gender of the individuals and the gestational timing of exposure to the famine. Lumey et al. [39] analyzed the DNA methylation status of two repetitive elements, long interspersed nucleotide element 1 (LINE-1) and satellite 2 DNA sequence (Sat2), in subjects with a history of prenatal exposure to the Dutch famine, as well as unexposed control subjects. The authors found that there were no significant differences in the DNA methylation status of the two repetitive elements between study subjects and control subjects. Based on these results, the authors suggested that there may not be any relationship between overall global DNA methylation and prenatal exposure to famine in adults with a history of such exposure, and only selected genes may be differentially methylated in such individuals [39].

Effect of postnatal nutrition on epigenetics in psychiatry

The nutrition of an individual during infancy, childhood, and adulthood can also influence his or her propensity to develop psychiatric disorders. In this regard, the nutrients most implicated in the pathogenesis of psychiatric disorders are those associated with one-carbon metabolism [40–43]. Although the psychiatric disorder most linked with the epigenetic effects of postnatal nutrition is schizophrenia, other psychiatric disorders such as major depressive disorder and bipolar disorder have also been associated with such effects [40–43].

Regarding the effects of postnatal nutrition on epigenetics in psychiatry, an interesting and important issue involves the gene encoding methylenetetrahydrofolate reductase (MTHFR), the enzyme that catalyzes the reduction of methylenetetrahydrofolate to MTHF. This gene is located on chromosome 1 at 1p36.3. The complementary DNA sequence is 2.2 kilobases long and comprises 11 exons [44].

Alternative splicing of the gene is known to occur. Two common polymorphisms linked with this gene involve two common alleles, *C677T* and *A1298C*. The *C677T* allele involves a point mutation at position 677 of the gene that changes a cytosine (C) base into a thymine (T) base, leading to an amino acid substitution (alanine for thymine) in the enzyme. Among *C677T* homozygotes, the activity of MTHFR is markedly reduced, leading to markedly increased levels of blood homocysteine levels. Among heterozygotes, the activity of MTHFR is reduced less, with lesser increases in homocysteine levels. Individuals who are homozygous for the *C677T* allele tend to have mildly increased blood homocysteine levels if their intake of folate is inadequate, but normal levels if their intake of folate is adequate [44]. The other common genetic variant involves the *A1298C* allele. In this allele, a point mutation in exon 7 results in an amino-acid substitution (glutamate for aspartate) in the enzyme. This allele, also referred to as the *C1289A* allele, is associated with decreased activity of MTHFR, but less than with the *C677T* allele. Several studies have examined an association between genetic polymorphisms in the gene encoding MTHFR and psychiatric disorders. Meta-analyses of such studies indicate that TT homozygotes at *C677T* have a significantly increased risk for schizophrenia, bipolar disorder, and major depressive disorder [45,46]. The *MTHFR 677TT* genotype is known to be modified by epigenetic mechanisms such as DNA methylation, and there is some preliminary evidence that such modifications can additionally influence the association of the *677TT* genotype with psychiatric disorders [47].

Epimutations involve heritable defects in gene expression [48]. Epimutations are of two types [49,50]. One type of epimutation is a primary, or "true," epimutation, where heritable defects of gene expression do not involve changes in the DNA sequence. This is the more common type of epimutation. The other type of epimutation is a secondary epimutation, which involves changes in the DNA sequence of genes that encode proteins involved in the epigenetic machinery of cells. In this context, the *MTHFR 677TT* genotype would qualify as a secondary epimutation involved in the pathogenesis of psychiatric disorders.

Transgenerational (heritable) effects of nutrition on epigenetics in psychiatry

There is evidence that environmental factors modifying epigenetic mechanisms of gene expression can be transmitted through the germ line from one generation to the next (i.e., inherited), a topic covered in detail in Chapter 28. These factors, which include nutrition, have to date been mainly conducted in plants and animals and not in humans [51]. For example, it has been shown by Burdge et al. [52] in rats that changes in the diet had transgenerational effects on the phenotype. These authors increased dietary energy by 25% at conception in F0 female rats and maintained it at this level to generation F3. They found altered DNA methylation of specific genes in a manner consistent with *de novo* epigenetic marks in each generation. At present, there is no evidence for transgenerational effects of nutrition on epigenetics in psychiatry [53].

Clinical applications of nutritional effects on epigenetics in psychiatry

Regarding the clinical applications of nutritional effects on epigenetics in psychiatry, what is currently of greatest interest is the use of the methyl donors, L-methylfolate and SAMe, in the prevention and

BOX 27.3 CLINICAL APPLICATIONS OF NUTRITIONAL EFFECTS ON EPIGENETICS IN PSYCHIATRY

- The methyl donors L-methylfolate and *S*-adenosylmethionine (SAMe) are being evaluated in major depressive disorder.
- Folic acid and vitamin B_{12} are being evaluated in schizophrenia.
- SAMe is being evaluated in Alzheimer's disease.

treatment of psychiatric disorders. L-methylfolate is chemically the same as MTHF, and, as referred to previously, is formed from folic acid, a reaction catalyzed by the enzyme MTHFR. L-methylfolate, unlike folic acid, is capable of crossing the blood–brain barrier and can function as a methyl donor [54,55]. Treatment with L-methylfolate, which is available as an over-the-counter supplement, appears to be safe and is relatively inexpensive [54]. SAMe, which functions as a methyl donor in the body, is now available commercially as salts that provide sufficient stability with superior quality throughout the shelf life of the product [56]. These salts are less degradable, are adequately absorbed after oral administration, and enter the brain in adequate amounts [56]. (See Box 27.3.)

Use of methyl donors in major depressive disorder

It is well known that a severe deficiency of vitamin B_{12} or folic acid can result in major depression [57–60]. Moreover, antiepileptic-induced folic acid deficiency is known to be associated with major depression [57,61]. Surveys conducted in the past have found that up to one-third of psychiatric patients had folic acid deficiency associated with the presence of major depression or dementia. These surveys also found that depressed patients who were treated with folic acid had significantly better clinical outcomes than the patients in whom the folic acid deficiency was not corrected [57]. Numerous single-blind [62,63], as well as randomized, double-blind [64,65], clinical trials have investigated the efficacy of SAMe in the management of major depression. Most of these studies have reported beneficial effects of SAMe in patients with major depression, and a meta-analysis of studies on the use of SAMe in the management of major depression has reported a beneficial effect of SAMe in the treatment of this disorder [66]. SAMe can be administered orally as well as parenterally for the treatment of major depression and may be useful as an adjunctive treatment with antidepressants in the treatment of this disorder [66].

In addition to SAMe, the efficacy of L-methylfolate in the management of major depression has also been investigated, although to a lesser extent. L-methylfolate is the active form of folic acid, and because it easily crosses the blood–brain barrier, it has a much better bioavailability than folic acid for the treatment of psychiatric disorders [67]. A retrospective comparison of L-methylfolate plus selective serotonin reuptake inhibitors (SSRIs) or selective noradrenaline reuptake inhibitors (SNRIs) at treatment initiation with SSRI or SNRI monotherapy from patient charts revealed that combined therapy using L-methylfolate was more effective in improving depressive symptoms [68]. More recently, two multicenter randomized, double-blind, parallel–sequential trials on the use of L-methylfolate as adjunctive therapy for antidepressant-resistant major depression were conducted [69]. The results suggested that L-methylfolate at a dose of 15 mg/day may be an effective, safe, and

relatively well-tolerated treatment strategy for patients who have a partial response or no response to treatment with SSRIs. L-methylfolate may be useful as an adjunctive treatment to treatment with antidepressants in patients with documented low levels of folic acid, and it may be useful in patients at a high risk for folic acid deficiency, such as those with alcoholism, pregnancy, gastrointestinal disorders, eating disorders, decreased levels of MTHFR, or high homocysteine levels, and those on drugs that can interfere with the conversion of folic acid to MTHF [55].

Use of methyl donors in major psychosis

The relationship between folic acid and vitamin B_{12} levels and the major psychoses (schizophrenia and bipolar disorder) are not as clear as they are with major depression [70–73]. However, there are case reports that suggest a causal relationship between vitamin B_{12} deficiency and major psychoses [71,74,75], and there is evidence that low levels of folic acid are associated with negative symptoms of schizophrenia [76]. In this light, a recent randomized multicenter trial investigated whether folic acid plus vitamin B_{12} supplementation benefited patients with chronic schizophrenia who were psychiatrically stable but displayed persistent symptoms despite antipsychotic treatment [77]. It was found that folic acid plus vitamin B_{12} improved negative symptoms significantly compared with placebo when genotype was taken into account, but not when genotype was not taken into account. Only patients homozygous for the *484T* allele of the gene encoding MTHFR showed significant improvement. Based on these results, it was suggested that folic acid and vitamin B_{12} can be used on a personalized basis to treat those patients with schizophrenia who have this genotype.

Use of methyl donors in cognitive disorders

Abnormalities in DNA methylation patterns of genes have been noted in postmortem brain obtained from patients with Alzheimer's disease (AD). West et al. [78] found hypomethylation of the amyloid precursor protein gene of an AD patient; however, hypomethylation of this gene in patients with AD has not yet been conclusively proved [79]. A recent study that investigated epigenetic changes in postmortem frontal cortex from AD patients found global DNA hypermethylation, but there was also DNA hypomethylation of many genes [80], which has been noted in patients with AD [81]. In this context, the use of SAMe in the treatment of AD is being investigated [79,81]. At present, studies investigating the use of SAMe in the management of AD are mainly in the preclinical stage [79].

Conclusions

Nutrition is an environmental factor that influences epigenetic mechanisms of gene expression. Nutrition is known to affect DNA methylation, histone modifications, and RNA-mediated regulation of gene expression. Nutritional effects on epigenetic mechanisms can take place prenatally and postnatally, and there is evidence that nutritional effects on epigenetics can be transmitted transgenerationally. Nutritional influences on epigenetic mechanisms are also thought to be of relevance to psychiatric disorders. Prenatal influences are exemplified by the effects of the Dutch Hunger Winter of 1944–45 and

the Great Chinese Famine of 1959–61. Nutritional influences on epigenetics in psychiatry can also occur postnatally, especially nutrients involved in one-carbon metabolism. Such influences can be affected by genetic mutations and polymorphisms of an individual, such as those in the gene encoding the enzyme MTHFR. At present, there is no evidence for transgenerational influences of nutrition on epigenetics in psychiatry. Nutritional influences on epigenetics in psychiatry may be put to use in the clinical management of patients by the use of methyl donors like SAMe and L-methylfolate in the management of patients with major depression, selected patients with schizophrenia, and possibly also patients with cognitive disorders such as Alzheimer's disease.

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Glossary

Epimutation Heritable defect involving epigenetic mechanisms of gene expression.
Major psychosis A type of psychiatric disorder that mainly includes schizophrenia and bipolar disorder.
One-carbon metabolism A metabolic pathway in the body centered around folic acid.
Primary epimutation Also referred to as true epimutation, it does not involve a change in the DNA sequence; more common than secondary epimutation.
Secondary epimutation Heritable defect in gene expression due to a change in the DNA sequence of a gene encoding a protein involved in the epigenetic machinery of a cell.

CHAPTER

Transgenerational (Heritable) Epigenetics and Psychiatric Disorders

28

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KEY CONCEPT

- Currently, there is no experimental evidence for trangenerational (heritable) epigenetic inheritance of psychiatric disorders in humans; however, there is experimental evidence for this phenomenon in animal models.

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ABBREVIATIONS

COMPASS Complex of proteins associated with Set1
CpG Cytosine–phosphate–guanine
DMRs Differentially methylated regions
DNMT DNA methyltransferase
endo-siRNAs Endogenous small interfering RNAs
H3K27me3 Trimethylation at lysine 27 on histone H3
H3K4me2 Dimethylation at lysine 4 on histone H3
IAP Intracisternal A particle
lncRNAs Long non-coding RNAs
LTR Long terminal repeat
miRNAs microRNAs
MSUS Unpredictable maternal separation and maternal stress
PGC Primordial germ cell
piRNAs PIWI-interacting RNAs
PND Postnatal day
PTMs Posttranslational modifications
TBT Tributyltin
TGF-β Transforming growth factor β
VECs Variably erased CpG islands

A novel non-Mendelian mechanism of inheritance

The transmission of hereditary information through genes, as described by the laws of Gregor Mendel, undoubtedly govern the current biological concept of modes of inheritance. However, recent evidence has suggested that an alternative mechanism for rapidly transmitting acquired states may also be acting to regulate the transcriptional landscape of cells across generations. This novel concept, termed transgenerational epigenetic inheritance, suggests that parts of the epigenome may be transmitted through the germ cell to subsequent generations, either by incomplete erasure during embryogenesis or by reinstatement of previously determined epigenetic marks. Epigenetic inheritance allows for environmental factors to act upon not only the individual exposed but also their offspring by providing a mechanism by which environmentally adapted epigenetic information may be transmitted through the germ line. This chapter will review the current evidence for epigenetic inheritance in animals and its role in psychiatric disease risk in humans.

Non-Mendelian patterns of expression of endogenous genes

There have been a limited number of cases of endogenous alleles that have been linked with non-Mendelian patterns of inheritance. Two of the best-studied transmissible epialleles (alleles that are differentially expressed in genetically identical individuals due to the epigenetic state of the gene) are the *agouti viable yellow* (A^{vy}) and *axin-fused* ($Axin^{Fu}$), discussed below. In mice, a small number of other metastable epialleles have been identified, all of which are the result of a recent insertion of a repetitive element or long terminal repeat (LTR) [1,2]. In humans, several putative metastable epialleles have been identified; however, due to the inter-individual epigenetic variation inherent in studying an outbred population, identification of such loci has been problematic [3].

Agouti viable yellow (A^{vy}) and axin-fused ($Axin^{Fu}$)

Transcription of the murine Agouti gene is generally initiated by a promoter in exon 2 of the *Agouti* (*A*) allele and results in the expression of a signaling molecule that causes follicular melanocytes to produce yellow pheomelanin pigment rather than black eumelanin pigment. The A^{vy} is the result of an intracisternal A particle (IAP) retrotransposon insertion upstream of the initiation site of

Agouti which contains a cryptic promoter in the proximal end [4,5] (Figure 28.1A). Ectopic *Agouti* expression results in yellow fur, diabetes, obesity, and tumorigenesis, and CpG methylation in the A^{vy} IAP negatively correlates with ectopic *Agouti* expression. Thus, the extent of methylation of this IAP correlates with coat colors ranging from yellow (unmethylated) to pseudoagouti (methylated) in inbred, isogenic mice (Figure 28.1B). The ectopic *Agouti* expression also correlates with increased body weight, such that mice with unmethylated IAPs are larger than those with methylated regions. IAPs appear to retain DNA methylation throughout erasure and reprogramming is normally occurring during early embryonic development [7,8]. Accordingly, the epigenetic state of this gene can be transmitted maternally; dams with a yellow coat phenotype have more yellow pups than agouti dams (Figure 28.1C) [4].

The methylation status of this IAP can be increased by providing pregnant dams with maternal dietary supplements of folic acid, vitamin B12, choline, and betaine [9,10] or with genistein (at levels comparable to humans consuming high soy diets) [11], thereby shifting offspring coat color distribution toward agouti and promoting obesity resistance. However, this environmentally induced shift in methylation is not transmitted by dams to their subsequent offspring [12].

A second metastable epiallele, $Axin^{Fu}$, encodes the Axin protein, which is expressed during both embryonic development and adulthood and acts to inhibit Wnt signaling, thereby having important effects on mammalian embryonic axis formation. Differential methylation at an IAP retrotransposon within intron 6 of the *Axin* gene ($Axin^{Fu}$) results in the presence (unmethylated) or absence (methylated) of a kinked tail [7]. The epigenetic state of this IAP, in contrast to the agouti-viable yellow allele, can be transmitted both maternally and paternally.

Exposure to environmental toxicants

The transgenerational effects of prenatal transient exposure to environmental toxicants were first investigated by Anway et al. [13] using the fungicide vinclozolin (an antiandrogenic compound). Prenatal exposure to vinclozolin was shown to have transgenerational effects on sperm number and viability and on infertility in males, and this effect was observed three generations downstream from the original exposure [13]. Since this pioneering study, several different environmental toxicants have been found to promote transgenerational epigenetic inheritance of adult-onset disease phenotypes, including plastics, pesticides, dioxin (TCDD), and hydrocarbons. For example, prenatal exposure to dioxin (F1) results in increased kidney disease in males and early-onset puberty, ovarian primordial follicle loss, and polycystic ovary disease in females [14]. Prenatal exposure (F1) to combinations of pesticides (permethrin and the insect repellant DEET), plastics (bisphenol A and phthalates), dioxin, and a hydrocarbon mixture (jet fuel, JP8) all result in spermatogenic cell apoptosis, early-onset female puberty, and reduced ovarian primordial follicle pool size [15].

Although much work has focused on endocrine disruptors and their influence on gonadal phenotypes, transgenerational effects have also been observed in relation to "obesogens," chemicals that directly or indirectly promote obesity by increasing the size or number of adipocytes or by altering the regulation of appetite and satiety [16]. Tributyltin (TBT) is a biocide that was once used for a variety of purposes including wood preservation and as a fungicide, but it is now thought to act as an obesogen. Prenatal exposure to TBT results in hepatic lipid accumulation and increases expression of genes involved in lipid storage and transport, lipogenesis, and lipolysis in the livers of mice exposed to the obesogen prenatally and two subsequent generations [16].

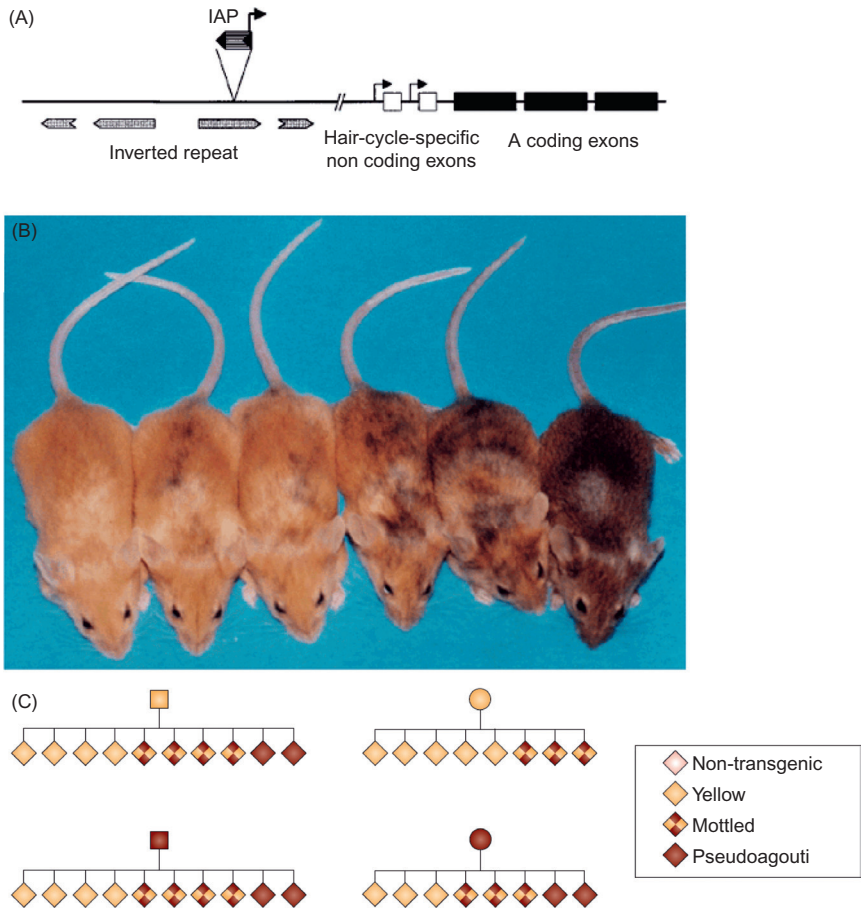

FIGURE 28.1

The *A^{vy}* allele. (A) Gene map showing the *A^{vy}* allele with the position of the intracisternal A particle (IAP) inserted in the pseudoexon 1A and the direction of transcription from the long terminal repeats (LTRs; arrowhead) in the opposite direction to that of the promotor regions. Hair-cycle-specific non-coding exons are indicated as open boxes, coding exons as filled boxes, and an interrupted inverted repeat as a gray bar arrow. Constitutive expression of agouti is caused by transcription initiation in a cryptic promoter (arrowhead) in the 3′ LTRs of the IAP in the *A^{vy}* allele. (B) The range of coat color phenotypes and body weights of isogenic C57Bl/6 *A^{vy}/a* mice can be seen here. These mice can range from yellow (left) through varying degrees of mottled yellow/agouti to completely agouti, referred to as pseudoagouti (right) because the mice are isogenic with fully yellow agouti. Yellow mice also have higher body weight than pseudoagouti mice. Pseudoagouti mice lack ectopic expression from the cryptic promoter due to methylation of the *A^{vy}*-associated IAP, while mice showing both yellow and agouti patches have a mosaic of cells that have or lack ectopic expression of the IAP [5]. (C) The phenotype of an *A^{vy}/a* sire does not affect the phenotype of the offspring when crossed with a congenic *a/a* female (left); however, the phenotype of the *A^{vy}/a* dam affects the phenotype of the offspring when crossed with a congenic *a/a* male. Yellow dams produce a higher proportion of yellow offspring than pseudoagouti dams, suggesting that the epigenetic state of the maternal *A^{vy}* locus is inherited in the offspring [6].

Metabolic influences

Diet has been shown to have persistent and transgenerational effects on a large number of metabolic processes. For instance, several genes involved in lipid and cholesterol biosynthesis were found to be regulated in the liver of the offspring of males fed a low-protein diet [17]. In addition, epigenomic profiling in the liver of offspring demonstrated widespread changes in cytosine methylation (~10–20%) and a significant and reproducible increase (~30%) in a likely enhancer for the key lipid regulator PPARA. The mechanism for this transmission has yet to be established; DNA methylation in sperm does not seem to be affected by a low-protein diet, but there is some evidence of altered chromatin posttranslational modifications.

Overfeeding also appears to have transgenerational effects on rodents, with both maternal obesity and neonatal overfeeding having persistent physiological effects on offpsring. Maternal obesity leads to an increase in body length and reduced insulin sensitivity through both the male and female lines up to the F2 generation [18]. However, in the F3 generation, only female offspring of the male line similarly display increased body length, and males from either the maternal and paternal line surprisingly demonstrate improved glucose tolerance, suggesting some adaptive or beneficial impact of the initial treatment. This complex pattern of sex-specific transmission has been often observed and may be related to differentially imprinted loci [19]. Similar to the effects of maternal obesity, neonatal overfeeding in mice, induced by limiting the litter size to four pups per dam during lactation, leads to obesity, insulin resistance, and glucose intolerance when adult [20]. The male offspring of these overfed mice, although not overfed themselves, develop fed and fasting hyperinsulimemia, hypertryglyceridemia, insulin resistance, and glucose intolerance, but not obesity, as adults. These mice then transmit a more mild phenotype of fasting hyperglycemia and glucose intolerance to their male offspring.

Human epidemiological studies support the evidence from animal studies suggesting that differential diet may have effects over multiple generations. Two retrospective cohort studies, the Dutch Hunger Winter families study and the Overkalix cohort, investigated the transgenerational effects of brief periods of food scarcity. In the Dutch cohort, the children born to mothers who had been exposed to famine *in utero* showed altered lipid profiles [21]. The children born to mothers who had been exposed to famine *in utero* also showed high neonatal adiposity and increased risk of metabolic disease, suggesting that the original prenatal exposure to famine may have transgenerational effects. The Overkalix cohort identified a critical period during early adolescence during which food scarcity correlated with survival of the grandchildren in a sex-specific manner; the paternal grandfathers' food supply was only associated with their grandsons' mortality risk ratio, while the paternal grandmothers' food supply was only associated with their granddaughters' mortality risk ratio [22].

Molecular mechanisms for epigenetic inheritance

Traditional thought, based on pioneering studies observing the epigenome of the developing embryo [23], suggested that epigenetic marks underwent complete erasure and reprogramming between generations, with large-scale DNA demethylation occurring immediately after fertilization and again after primordial germ cell migration. This concept of complete reprogramming was a key point in the field of epigenetics until the early 1980s, when it was discovered that a small subset of genes, termed imprinted genes, escape reprogramming during embryogenesis. In the late 1990s, another group of genes, called metastable epialleles, were also shown to escape reprogramming during both

gametogenesis and embryognesis. Currently, many mechanisms have been suggested for the transfer of epigenetic information across generations, including DNA methylation, histone posttranslational modifications (PTMs), non-coding RNAs, and the DNA sequence itself.

DNA methylation

Although it is clear that after fertilization DNA methylation undergoes two important periods of erasure and reprogramming during development, we are also becoming increasingly aware that some sequences appear to be resistant to epigenetic reprogramming, allowing for possible transmission of epigenetic information across generations. These resistant loci include imprinted differentially methylated regions, IAPs, and recently identified variably erased CpG islands (VECs) (Figure 28.2).

Parental imprinting provided the first examples of genes where DNA methylation patterns remained through embryogenesis. Imprinted genes are expressed mono-allelically, dependent on the parent-of-origin of the allele, with maternal and paternal alleles in different transcriptional and epigenetic states in the adult. The current number of imprinted loci in the brain appears to be more than 1300, much higher than originally thought, with several hundred genes showing parental expression

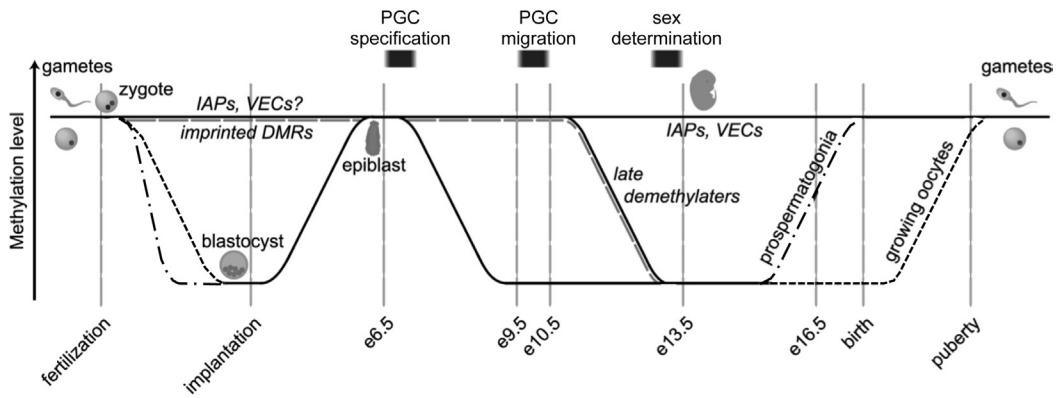

FIGURE 28.2

DNA methylation erasure and reprogramming during early development. After fertilization, the maternal genome (black dotted line) undergoes passive demethylation and the paternal genome (black dash-dot line) undergoes an active form of rapid demethylation, after which follows a period of *de novo* methylation occurring after implantation (black solid line). Primordial germ cells (PGCs) are specified during the epiblast stage (shading at top of figure), and this methylation has to again be reset in these cells. Thus, from e6.5 the figure shows the methylation changes in germ line cells only. The large majority of sequences are demethylated in PGCs by e9.5; however, there is a subset of sequences, termed late demethylaters, which includes imprinted genes that are reprogrammed only after PGC migration (grey dashed line). IAPs are sequences that are resistant to demethylation both postfertilization and during PGC reprogramming. In addition, variably erased CpG islands (VECs) can resist erasure during PGC reprogramming. Currently, the methylation dynamics of VECs during postfertilization reprogramming is not known. After sex determination, *de novo* methylation of germ cells occurs in a sex-specific manner. In prospermatogonia, methylation is complete during the prenatal period, while in oocytes methylation is established postnatally during the growth phase [24].

bias [25,26]. Thus, imprinting is likely an important mechanism of gene regulation in the brain and may have critical implications for psychiatric disease risk. However, because this form of epigenetic memory depends on the sex of the individual, and thus only lasts one generation, it is not traditionally listed among the forms of transgenerational epigenetic inheritance. That said, mechanisms that allow imprinted genes to transmit epigenetic information across generations may also be similarly employed to transmit epigenetic marks in a multigenerational manner.

A recent study has proposed that DNA methylation can be resistant to reprogramming at many CpG islands in the mouse primordial germ cell (PGC), pointing to a possible mechanism for transgenerational epigenetic inheritance. Seisenberger et al. [27] have identified particular sequences, variably erased CpG islands (VECs), that variably escape reprogramming at multiple stages of development. These regions are not associated with IAPs and differ from CpG islands associated with IAPs, which remain methylated in all developmental stages. These IAP-associated CpG islands occur rarely, with the majority of IAPs being more frequently associated with resistant non-CpG island promoters. More VECs were found in sperm than in oocytes, suggesting a potential bias in the male germ line for maintenance of methylation through erasure.

Aberrant DNA methylation in the germ line has been suggested to be a possible mechanism for epigenetic inheritance of environmentally induced disease. Several studies have indeed demonstrated differentially methylated regions (DMRs) in the sperm of the progeny of rats exposed to environmental toxicants. MeDIP–ChIP in the sperm of F3 rats whose F0-generation mothers were exposed to vinclozolin identified 52 different regions with statistically significant altered DNA methylation, confirming that environmental toxicants can alter DNA methylation in the germ line [28]. Within these regions a consensus DNA sequence was identified in 75% of the promoters, but only 17% of a random set of promoters contained this same motif, providing some suggestion that particular DNA sequences may be more likely to display differential methylation in sperm in a transgenerational manner. Additionally, a copy number variant in a candidate promotor was also identified, suggesting that changes in the germ line epigenome may promote genetic abnormalities in subsequent generations. Fifty DMRs in the sperm of F3 rats whose F0-generation mothers were exposed to dioxin and 197 DMRs in the sperm of F3 rats whose F0-generation mothers were exposed to a combination of plastics-derived endocrine disruptors have also been described, again confirming that environmental toxicants can promote epimutations in the germ cell several generations after exposure [14,29].

It has also been suggested that, although DNA methylation patterns may be in part transmitted across generations, they may not be the critical mark required for phenotypic transmission to occur. One piece of evidence supporting this is that epigenetic marks can be inherited during meiosis in fission yeast, which do not have DNA methylation [30]. Additionally, in humans, the level of DNA methylation is correlated within families; however, the precise methylation patterns are not identical across generations or between tissues, prompting the suggestion that DNA methylation is a secondary mark, not the primary mechanism for epigenetic inheritance [31]. In fact, following maternal transmission, DNA methylation of A^{vy} is not present in the blastocyst, suggesting that DNA methylation is not the critical inherited mark in the case of the agouti mouse [4]. This points to histone modifications as a possible alternative, as discussed below.

Posttranslational modifications on chromatin

Although histone posttranslational modifications (PTMs) are conventionally seen as more transient than DNA methylation, recent findings have demonstrated that histone PTMs may also be

transmitted through the germ line. Although the large majority of histones are cleared and replaced by proteasomes in mature sperm, approximately 4% of the haploid genome in humans and 1–2% in mice remains packaged into nucleosomes [32,33]. Genome-wide studies in humans and mice have demonstrated that some genes (particularly those that are not expressed during gametogenesis or embryogenesis) maintain a repressive histone mark, histone 3 lysine 27 trimethylation (H3K27me3), at their promoters [32,33]. However, due to the dynamic nature of chromatin it is possible that the re-establishment of transcriptional silencing during early development is so rapid that the erasure and reprogramming have not been detected [6].

Studies using *C. elegans* as a model have suggested that H3K4 methylation may play a key role in transgenerational epigenetic inheritance; both epigenetic inheritance of sterility and longevity have been reported to involve this mark [34,35]. Mutants of *spr-5*, a worm ortholog of a histone demethylase that acts on H3K4 dimethylation (H3K4me2) have immediate and progressive decreased brood size and eventual progeny sterility, suggesting that failure to reset H3K4me2 at particular genes in the germ line in PGCs can result in progressive transgenerational sterility [34]. Epigenetic inheritance of longevity has also been described in *C. elegans* using genetically wild-type descendants from ancestors mutant for members of the COMPASS–H3K4 methylation complex [35]. This complex (containing ASH2, WD repeat-containing protein 5, and SET2 in worms) is conserved across species and catalyzes the trimethylation of a mark of active transcription, H3K4me3. Interestingly, manipulation of alternative longevity-promoting pathways or other chromatin regulators did not display the same transgenerational effect on longevity, again suggesting that H3K4 may play a key role in epigenetic inheritance of complex traits.

RNAs (RNA, miRNA, piRNA)

Mature sperm express RNA populations, and sperm-borne RNA has been detected in the zygote [36], providing one possible mechanism for the transfer of non-genetic information from sperm to embryo. Both sperm and oocytes express mRNAs, long non-coding RNAs (lncRNAs) and small RNAs that may be involved in gene silencing, including microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs), and PIWI-interacting RNAs (piRNAs). In particular, small non-coding RNAs have been investigated as a mechanism for transgenerational epigenetic inheritance.

One of the first examples of a possible role for miRNAs came from a mouse with a transgene inserted downstream from the *Kit* promoter. This insertional mutation results in a white-tail phenotype that demonstrates non-Mendelian inheritance; many wild-type offspring of the *Kit* mutant mice retain the mutant phenotype (Kit*), and this can be transmitted to the next generation [37]. Mice heterozygous for the *Kit* mutation and Kit* males both have high RNA levels in sperm, and the Kit* phenotype could be induced by microinjection of miRNAs targeted to the *Kit* locus. Transgenerational inheritance of cardiac hypertrophy or increased body size can also be induced by injecting fertilized eggs with specific miRNAs targeting *Cdk9* or *Sox9*, respectively [38,39]. Neither *Kit* nor *Sox9* RNA-mediated epigenetic heredity is observed in DNMT2 knockout mice, suggesting that DNMT2 may have a critical role in mediating this paramutation-like mechanism [40].

In *C. elegans*, *Drosophila*, and *Oxytricha trifallax*, piRNAs can trigger germ line gene silencing in a stable multigenerational manner [41,42]. In *C. elegans*, piRNAs can initiate a persistent silencing lasting at least 20 generations that is independent of the original piRNA trigger, but dependent on a subsequently induced nuclear RNAi/chromatin pathway [42]. In this experiment, there was no

redundancy in the system, with both small RNA and chromatin factors being required for transgenerational epigenetic inheritance [42]. In *Drosophila*, piRNAs have been shown to be maternally inherited through the cytoplasm [43]. Mouse spermatogenesis also involves a complex regulation of piRNAs, providing a possible substrate for epigenetic inheritance [44].

Paternal genome

Animal studies have reported cases where the paternal genome confers disease risk on progeny independent of the transmitted genomic information, suggesting that genotype can promote epigenetic instability at particular loci. The *Obrq2a*$^{A/J}$ allele in the paternal or grandpaternal generation can confer obesity resistance by inhibiting diet-induced obesity and reducing food intake in normally obesity-susceptible C57Bl/6J mice fed a high-fat diet [45]. Interestingly, the obesity-resistant phenotypes can be transmitted through the paternal line, independent of inheriting the initially responsible *Obrq2a*$^{A/J}$ allele, and when social interaction between the sire and either his offspring or the pregnant dam is removed. This suggests that the transmission occurs through the male germ line through an epigenetic mechanism and provides further evidence that body weight and food intake may be influenced by epigenetic inheritance.

A hypomorphic mutation of *Mtrr*, a gene that encodes a protein responsible for the appropriate functioning of folate and methionine cycles in mice, disrupts folate metabolism and has transgenerational effects on offspring development that are independent of genotype [46]. *Mtrr* deficiency in either maternal grandparent can result in congenital malformations in their grandprogeny irrespective of their maternal environment or wild-type genotype, suggesting that epimutations resulting from the original *Mtrr* deficiency are not erased between generations.

Epigenetic inheritance of psychiatric disorder-related behavior in animal models

Although inbred mice are genetically identical, they still show a wide range of complex behaviors that may be due to differences in epigenetic marks. Several lines of evidence stemming from animal models have suggested that these differences in epigenetic marks may be inherited, in part because there are a number of examples of behavioral transmission through the paternal line despite rodent offspring having only environmental contact with their dams. Activity levels in a novel environment have been associated with anxiety-like behavior in mice; such behavior is correlated between sires and male offspring in genetically identical mice, suggesting non-genetic mechanisms of inheritance [47]. In addition to transmission of innate anxiety-like traits, transmission of acquired complex behaviors has also been observed in mice and will be focused on here.

Stress-induced behavior

Chronic stress experiences occurring at any stage of development can have profound and persistent effects on behavior, and in pathological cases can develop into psychiatric disorders including anxiety disorders and depression. These disorders are often present in the progeny of those originally diagnosed, and this transmission cannot be explained by parental factors alone, opening up the intriguing possibility that an environmentally induced psychiatric disorder may be epigenetically transmitted

to offspring. Several animal studies have aimed at investigating this putative epigenetic transmission using prenatal, postnatal, and adult stress models.

One study in the mouse has demonstrated that chronic variable stress during early gestation (gestation days 1 to 7) results in dysmasculinization and increased stress sensitivity in the male offspring exposed to the prenatal stress, and to their subsequent male progeny [48]. The offspring of males exposed to early prenatal stress display, similar to females, an increase in depressive-like behaviors in the tail suspension test compared with their male controls. They also have shorter anogenital distance and testis weight, further showing physiological evidence of dysmasculinization. At a molecular level, brains taken from these male offspring at postnatal day 1 (PND1) demonstrate a feminization in gene and miRNA expression. Specifically, three miRNAs (miR-322, miR-574–3p, and miR-873) that target a member of the transforming growth factor β (TGF-β) family that regulates the release of gonadal hormones are reduced similar to female levels, providing a neurodevelopmental mechanism for the dysmasculinization observed in these mice.

Early postnatal development in rodents is a key period of maturation in rodents and may be a stage when they are particularly susceptible to stressful insults. Maternal separation in combination with unpredictable maternal stress (MSUS) is a model designed to create a stressful and unpredictable postnatal environment for mouse pups throughout their early development [49]. MSUS pups are separated from their dams for 3 hours each day (PND1–14) at unpredictable time points. During this separation period, dams are additionally exposed to a further unpredictable stressor. This treatment results in a complex behavioral phenotype of impulsive-like and depressive-like behaviors in adults exposed to the postnatal treatment. Aspects of this perturbed phenotype can be transmitted in a sex-specific manner through both the male and female line, even when the offspring of the females exposed to the postnatal stress are raised by undisturbed control females [49,50]. These findings strongly suggest an epigenetic transmission of negative behavioral traits resulting from exposure to stressful environments. In support of this, sperm of males exposed to MSUS and the brain and sperm of their offspring have similar DMRs in the *crfr2*, *mecp2*, and *cb1* genes, suggesting transmission of aberrant DNA methylation patterns through the male germ line [49].

Several aspects of social behavior were also perturbed across generations as a result of the initial MSUS treatment [51]. MSUS males and their female offspring (F2 and F3 MSUS) had deficits in social recognition memory. An additional interesting finding was that although males exposed to the postnatal stress did not show social anxiety, they transmitted enhanced social anxiety to their F2 and F3 male progeny. This suggests a complex and sex-specific transmission of social deficits resulting from postnatal stress. Although postnatal stress induced some deficits in social behavior, it may also be associated with some positive adaptations, as adult F2 MSUS males were more resilient to social defeat experiences. These experiments provided the first evidence of a positive adaptation provided by a likely epigenetic inheritance mechanism.

Another model of infant maltreatment, where infant rats are exposed to a stressed and "abusive" dam 30 minutes per day during the first postnatal week, demonstrated decreased BDNF mRNA and increased methylation in *BDNF* exons IV and IX in the prefrontal cortex when adult [52]. The offspring of females exposed to postnatal stress cross-fostered to untreated females also showed similar altered methylation patterns, suggesting that an epigenetic mechanism, and not postnatal environment, determines transmission of this DMR.

Male mice exposed to social defeat during adulthood transmit several depressive-like and anxiety-like behaviors to their offspring [53]. When IVF is performed with sperm from defeated males, the

majority of these behavioral deficits are no longer observed, suggesting that dams may rear offspring from stressed males differently than normal offspring or that IVF conditions may alter epigenetic marks present on the sperm from these animals. However, using IVF conditions, offspring of stressed males adopt a floating posture more quickly in the forced swim test, providing evidence that even stress occurring during adulthood may have transgenerational effects on depressive-like behaviors via epigenetic mechanisms.

Chronic social instability during adolescence and adulthood in mice (PND27–76) results in anxiety-like behavior in females (as assessed using the open field and elevated plus maze) and social avoidance in both males and females (F0) [54]. Interestingly, F1 females and F2 and F3 female offspring from the paternal line also displayed similar anxiety-like behavior and social deficits, even when sires did not display behavioral deficits themselves. Thus, similar to social anxiety behaviors observed in MSUS mice, males with a history of chronic social instability can act as "silent" carriers of behavioral deficits likely resulting from epigenetic mechanisms (Figure 28.3).

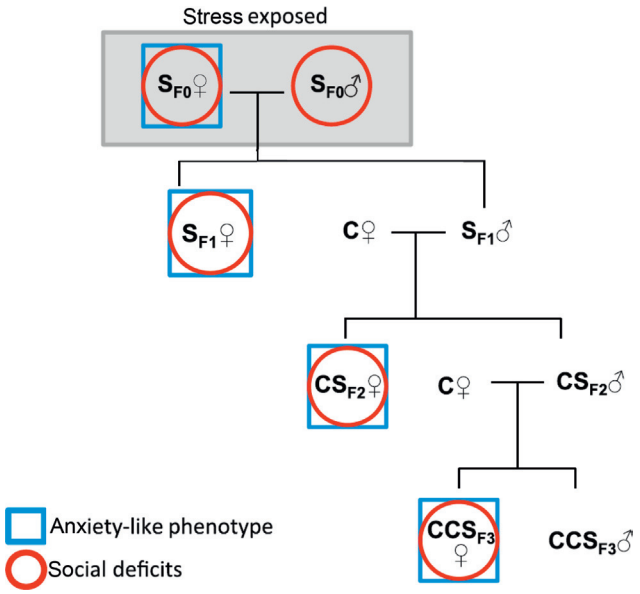

FIGURE 28.3

Transmission of the effects of chronic social instability in mice. A model of chronic social instability was performed during adolescence and adulthood, and stressed males and females (S_{F0}) were mated to generate offspring (F1). The F2 generation was generated by mating F1 male offspring of stressed mice to control non-stressed females (C). F2 male mice were then mated with control female mice to generate the F3 generation. A square indicates an increase in anxiety-like behavior, and a circle indicates social deficits compared with control mice. F1 females and F2 and F3 female offspring from the paternal line display anxiety-like behavior and social deficits. Thus, male mice with a history of stress can act as silent carriers, passing down behavioral deficits to their female offspring.

Adult drug use

The role of epigenetic inheritance in drug use has not been well studied. However, it was shown that *DNMT1* expression is decreased in the seminiferous tubules of the testes following cocaine self-administration, suggesting that epigenetic mechanisms may be disturbed at the level of the germ line as a result of cocaine usage [55]. In addition, there are several examples of paternal cocaine use affecting the behavior of their progeny in a sex-specific manner. For example, paternal cocaine administration results in hyperactivity and increased perseveration in the T-maze in male offspring [56] and deficits in working memory performance in female offspring [55]. Interestingly, in terms of drug addiction and drug-seeking behaviors, cocaine-experienced sires produced male offspring that were less willing to work for cocaine reward than normal rats, suggesting a decreased reinforcing effect of cocaine in these animals [57]. This contradicts human epidemiological data suggesting that cocaine addiction is heritable, indicating that environmental factors may play a larger role in human situations.

In rats, the transmitted cocaine-resistant phenotype was associated with increased acetylated histone H3 on *BDNF* promoters I, IV, and VI in the sperm of cocaine-exposed rats, and an apparent partial transmission to the brain of their male offspring; increased acetylated histone H3 on *BDNF* promoter IV was also observed in the medial prefrontal cortex of their male progeny [57]. This increased acetylation was linked functionally to increased expression in the exon IV-containing transcript of *BDNF* and increased BDNF protein in the male offspring of cocaine-experienced sires.

Conclusion

Genetic information is clearly the main mechanism for inheritance; however, it is becoming increasingly evident that epigenetic mechanisms also contribute to heritable phenotypes. Having said that, the extent to which transgenerational epigenetic inheritance occurs is still unclear. Currently, the number of alleles where the epigenetic state is inherited across generations remains limited and often shows incomplete penetrance, with only a proportion of the offspring being affected. Indeed, this could also be said about the environmentally induced behaviors that show transmission, with offspring being affected in an often complex sex-specific manner and frequent incomplete penetrance being observed. However, transgenerational epigenetic inheritance has now been observed for a number of different phenotypes, including exposure to environmental toxicants, diet, and stress, and has been demonstrated in a variety of species. This suggests that the phenomenon may play a significant role in the heredity of traits and that transgenerational epigenetic inheritance may have been selected during evolution to allow an individual the obvious advantage of passing on information about one's environment to its offspring.

The mechanisms underlying transgenerational epigenetic inheritance remain unclear and hinge on the determination of how epigenetic changes at particular loci are maintained in the germ line, or alternatively how the epigenetic marks or signals are erased and reset [24]. Evidence for both circumstances has been provided, with particular loci escaping epigenetic reprogramming and with the demonstration that certain non-coding RNA can transfer from the germ cell to the zygote and can impact reprogramming after fertilization. Addressing questions regarding the mechanism for epigenetic inheritance will remain key to further elucidating the contribution of transgenerational epigenetic inheritance to psychiatric disease risk and onset.

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Glossary

Dam Female parent.
F (filial) A generation in a breeding experiment that is successive to a parental generation.
Germ cell Reproductive cell in the body; may be a sperm or ovum.
Germ line The cellular lineage from which eggs and sperm are derived.
Sire Male parent of a quadruped.

Abbreviations

11β-HSD	11β-Hydroxysteroid dehydrogenase
3C	Chromosome conformation capture
5-aza-CdR	5-Aza-2′-deoxycytidine
5-aza-CR	5-Azacytidine
5-aza-dC	5-Aza-2′-deoxycytidine
5-C	5-Cytosine
5caC	5′-Carboxylcytosine
5-CH_3-THF	5-Methyltetrahydrofolate
5-fC	5-Formylcytosine
5-gmC	5-Glucosylmethylcytosine
5-HIAA	5-Hydroxyindoleacetic acid
5hmC/5-hmC	5-Hydroxymethylcytosine
5hmU	5′-Hydroxyuracil
5hmU	5-Hydroxymethyluridine
5-HT	Serotonin
5-HTT	Serotonin transporter
5-HTTLPR	Serotonin-transporter-linked polymorphic region
5-LOX	5-Lipoxygenase
5mC/5-mC	5-Methylcytosine
5mdC	5-Methyl-2′-deoxycytidine
Aβ	Amyloid-beta peptide
ABN	Arched-back nursing
Ac	Acetylation
ACC	Anterior cingulate cortex
ACF1	ATP-utilizing chromatin assembly and remodeling factor 1
aCGH	Array comparative genomic hybridization
ac-H3K9K14	Acetylated histone H3 at lysines 9/14
ACTB	Actin, beta
ACTH	Adrenocorticotropic hormone
ACTL6A/B	Actin-like 6A/B
AD	Alzheimer's disease
ADD	ATRX–DNMT3–DNMT3L
ADHD	Attention deficit hyperactivity disorder
AGRE	Autism Genetic Resource Exchange Consortium
AID	Activation-induced deaminase
AID/APOBEC	Activation-induced deaminase/apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
AKT	Atypical kinase
ALC	Acetyl-L-carnitine
ALS	Amyotrophic lateral sclerosis
ALSPAC	Avon Longitudinal Study of Parents and Children
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-2	Activating protein 2
APOBEC	Apolipoprotein B mRNA-editing enzyme complex

apoD	Apolipoprotein D
APP	Amyloid precursor protein
Arc	Activity-regulated cytoskeleton-associated protein
ARID	AT rich interactive domain
ARIES	Accessible Resource for Integrated Epigenomics Studies
AS	Angelman syndrome
Ascl1	Achaete–scute complex homolog 1
ASD	Autism spectrum disorder
ASE	Allele-specific expression
ASH2L	ASH2-like
ASOs	Antisense oligonucleotides
AST	Antisense transcript
ASXL1	Additional sex combs like 1
ATP	Adenosine triphosphate
ATP10C	ATPase, Class V, type 10C
ATRX	Alpha thalassemia X-linked mental retardation
AVP	Arginine vasopressin
AXAS	ASD, XLID, ADHD, SZ
BACE1	β-site APP cleaving enzyme 1
BAF	Brg1-associated factor
BBB	Blood–brain barrier
BD	Bipolar disorder
BDI	Beck Depression Index
BDNF	Brain-derived neurotrophic factor
BER	Base excision repair
BGT	beta-Glucosyltransferase
bHLH	Basic helix–loop–helix
BIS-seq	Bisulfite sequencing
BLA	Basolateral amygdala
BMP	Bone morphogenetic protein
BOLD	Blood oxygenation level dependent
BP	Bipolar disorder
BPA	Bisphenol-A
BPD	Borderline personality disorder
Brg1	Brahma-related gene 1
BWS	Beckwith–Wiedeman syndrome
C	Cytosine
CaMK	Ca^{2+}/calmodulin-dependent kinase
CaMKIIα	Calcium-dependent kinase IIα gene
CaN	Calcineurin
CAPN6	Calpain 6, dipeptidyl-peptidase 10
CAR	Cortisol awakening response
CB1	Endocannabinoid receptor 1
CBP	CREB-binding protein
CC	Collaborative Cross
Cdk5	Cyclin-dependent kinase-5 gene
CDKL5	Cyclin-dependent kinase-like 5

Cdkn1c	Cyclin-dependent kinase inhibitor 1c gene
Cdkn3	Cyclin-dependent kinase inhibitor 3 gene
CeA	Central amygdala
CEBPA	CCAAT/enhancer-binding protein alpha
c-*fos*	FBJ murine osteosarcoma viral oncogene homolog
CGG	Cytosine–guanine–guanine
CGRA	Combined glycosylation restriction analysis
CHAPs	Cyclic hydroxamic acid-containing peptides
CHARGE	Coloboma, heart anomaly, atresia of the nasal choanal, retardation, and genital and ear anomalies
CHARM	Comprehensive high-throughput array for relative methylation
CHD	Chromodomain helicase DNA-binding protein
ChIP	Chromatin immunoprecipitation
ChIP-chip	Chromatin immunoprecipitation coupled with promoter microarray
ChIP-seq	Chromatin immunoprecipitation sequencing
CHRNA2	Cholinergic receptor, nicotinic, alpha 2
cKO	Conditional knockout
CM	Childhood maltreatment
CMS	Cytosine-5-methylenesulfonate
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CNV	Copy number variation/variant
COBRA	Combined bisulfite restriction analysis
COMPASS	Complex of proteins associated with Set1
COMT	Catechol-*O*-methyltransferase
CON	Control
COPG2	Coatomer protein complex, subunit gamma 2
COREs	Clusters of open regulatory elements
CORT	Corticosterone
COX2	Cyclooxygenase 2 gene
CpG	Cytosine–phosphate–guanine
CPN	Central pretectal nucleus
CPP	Conditioned place preference
CPu	Caudate putamen
CR	Caloric restriction
CRE	cAMP response element
CREB	cAMP response/responsive element-binding protein
CREBBP	CREB-binding protein
CREST	Ca^{2+}-responsive transactivator
CRF	Corticotropin-releasing/release factor
CRH	Corticotropin-releasing hormone
CRISPR	Clustered regularly interspaced short palindromic repeats
CRYM	Crystallin, mu
CS	Conditioned stimulus
CSF	Cerebrospinal fluid
CTCF	CCCTC-binding factor
CTCL	Cutaneous T-cell lymphoma

Ctip2	COUP-TF interacting protein 2
CUL4B	Cullin 4B
CUMS	Chronic unpredictable multiple stress
CVS	Chronic variable stress
CXXC1	CXXC finger protein 1
CYC1	Cytochrome C-1
CYP2E1	Cytochrome P450 2E1
D_1	Dopamine receptor subtype 1
D_2	Dopamine receptor subtype 2
DA	Dopamine
DAO	D-amino-acid oxidase
DAOA	D-amino acid oxidase activator
DARPP-32	Dopamine- and cAMP-regulated neuronal phosphoprotein of 32 kDa
DAT	Dopamine transporter
dATP	Deoxyadenosine triphosphate
DAXX	Death-domain associated protein
DBP	D-binding protein
DDC	L-DOPA-decarboxylase
ddNTPs	Dideoxynucleotides
DEAD/H	Asp–Glu–Ala–Asp box helicase
DG	Dentate gyrus
DGCR8	Di-george critical region 8 gene
DHA	Docosahexaenoic acid
DISC1	Disrupted in schizophrenia 1 gene
DIV	Days *in vitro*
DL	Deep layer
DLB	Dementia with Lewy bodies
DLPFC	Dorsolateral prefrontal cortex
Dlx2	Distal-less homeobox 2 gene
Dm-20	Neuronal membrane glycoprotein M6-b-like
DMEM	Dulbecco's Modified Eagle Medium
DMP	Differentially methylated position
DMR	Differentially methylated region
DNAm	DNA methylation
DNMT	DNA methyltransferase
DNMT1	DNA methyltransferase 1
DNMTi	DNA methyltransferase inhibitor
dNTP	Deoxynucleotide triphosphate
DOHaD	Developmental origins of health and disease
DOPAC	3,4-Dihydroxyphenylacetic acid
DPY30	Dpy-30 homolog
DRD1	Dopamine receptor 1 gene
DRD2	Dopamine type-2 receptor
DRD4	Dopamine receptor gene, type 4
DRPLA	Dentatorubral–pallidoluysian atrophy
dsRNA	Double-stranded RNA
DTNBP1	Dystrobrevin-binding protein 1

DZ	Dizygotic
E2	Estradiol
E7	Embryonic day 7
E11	Embryonic day 11
E33	Embryonic day 33
EAAT2	Excitatory amino acid transporter 2
ECT	Electroconvulsive therapy
EE	Environmental enrichment
Eed	Embryonic ectoderm development
EED/WAIT1	Embryonic ectoderm development polycomb histone-methylation modulator
EGR1	Early growth response 1
EGR3	Early growth response 3 gene
EHMT	Euchromatin histone methyltransferase
ELA	Early-life adversity
ELISA	Enzyme-linked immunosorbent assay
ELN	Elastin
ELS	Early-life stress
ENCODE	Encyclopedia of DNA Elements
endo-siRNAs	Endogenous small interfering RNAs
ePCR	Emulsion PCR
EPDS	Edinburgh Postnatal Depression Scale
eQTLs	Expression quantitative trait loci
ER	Estrogen receptor
ERBB4	v-erb-A avian erythroblastic leukemia viral oncogene homolog-like 4 gene
ERK	Extracellular signal-regulated kinase
ERRg	Estrogen-related receptor gamma
ESC	Embryonic stem cell
Eset	ERG-associated protein with SET domain
EWAS	Epigenome-wide association study
EZH2/Ezh2	Enhancer of zeste homolog 2
FAAH	Fatty acid amide hydrolase
FACS	Fluorescence-activated cell sorting
FAIRE-seq	Formaldehyde-assisted isolation of regulatory elements
FAM126A	Member A of family with sequence similarity 126
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FGF2	Fibroblast growth factor 2
FISH	Fluorescence *in situ* hybridization
FKBP5	FK506-binding protein 5
FMR1	Fragile X mental retardation gene 1
fMRI	Functional magnetic resonance imaging
FMRP	Fragile X mental retardation protein
FRET	Fluorescence resonance energy transfer
FUDR	5-Fluoro-2′-deoxyuridine
FXS	Fragile X syndrome
FXTAS	Fragile X-associated tremor/ataxia syndrome
GA	Gestational age

GABA	gamma-Aminobutyric acid
$GABA_A$	gamma-Aminobutyric acid A
GABR	$GABA_A$ receptor subunit
GAD	Glutamic acid decarboxylase
GAD1	Glutamate decarboxylase 1
GAD67	Glutamate decarboxylase 67
GADD45	Growth arrest and DNA damage-inducible 45
Gadd45b	Growth arrest and DNA-damage-inducible 45 beta gene
GC	Glucocorticoid
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GLIB	Glucosylation, periodate oxidation, biotinylation
GLP	G9a-like protein
GLT1	Glutamate transporter 1
GNAT	GCN5 *N*-acetyltransferase
Gp130	Glycoprotein 130
GPNMB	Transmembrane glycoprotein NMB gene
GR	Glucocorticoid receptor
GREs	Glucocorticoid response elements
GRIA2	Ionotropic AMPA glutamate receptor subunit 2 gene
GRM	Metabotropic glutamate receptor
GRM1	Glutamate receptor, metabotropic 1
GRM2	Glutamate receptor, metabotropic 2
GSK3	Glycogen synthase kinase 3
GWAS	Genome-wide association study/scan
H2A	Histone 2A
H3K4	Histone H3 lysine 4
H3K4me2	Dimethylation at lysine 4 on histone H3
H3K4me3	Trimethylation at lysine 4 on histone H3
H3K9	Histone H3 lysine 9
H3K9me2	Dimethylation at lysine 9 on histone H3
H3K9me3	Trimethylation at lysine 9 on histone H3
H3K27	Histone H3 lysine 27
H3K27me3	Trimethylation at lysine 27 on histone H3
H3K36	Histone H3 lysine 36
H3K79	Histone H3 lysine 79
H3K9me	Histone H3 lysine 9 methylation
H3S10	Histone H3 serine residue 10
HAT	Histone acetyl transferase
HCFC1	Host cell factor C1
HCG9	Human leukocyte antigen complex group 9 gene
HCY	Homocysteine
HD	Huntington's disease
HDAC	Histone deacetylase
HDAC1	Histone deacetylase 1
HDACi	Histone deacetylase inhibitor

HDM	Histone demethyltransferase
HDMT	Histone demethyltransferase
HELP	HpaII tiny fragment enrichment by ligation-mediated PCR
hERG	Human ether-a-go-go related gene
HES	Hairy and enhancer of split
HGP	Human Genome Project
HIST1H2BC	Histone cluster 1, H2bc
HIST1H2BD	Histone cluster 1, H2bd
HIST1H2BG	Histone cluster 1, H2bg
HIST1H2BH	Histone cluster 1, H2bh
HIST1H4K	Histone cluster 1, H4k
HIST2H2BE	Histone cluster 2, H2be
HLA	Human leukocyte antigen
Hmc	Hydroxymethyl cytosine
hMeDIP	Hydroxymethylated MeDIP
HMT	Histone methyltransferase
HP1	Heterochromatin protein 1
HPA	Hypothalamic–pituitary–adrenal
HPC	Hippocampus
HPLC	High-performance liquid chromatography
HR	High responder
HRM	Heterozygous *reeler* mouse
HRM	High-resolution melt
HS	Hypersensitive
HTR2A	5-Hydroxytryptamine (serotonin) receptor 2A, G protein-coupled
HTR2A	Serotonin receptor 2A gene
HTR2C	Serotonin receptor 2C gene
HTT	Huntingtin
HVA	Homovanillic acid
IAP	Intracisternal A particle
IC	Imprinting center
ICF1	Immunodeficiency-centromeric instability 1
Id	Inhibitor of differentiation
ID	Intellectual disability
IEGs	Immediate early genes
IGF2	Insulin-like growth factor 2
Igf2	Insulin-like growth factor 2 gene
IL-1α	Interleukin 1α
IL-6	Interleukin 6
IL-11	Interleukin 11
IMGSAC	International Molecular Genetic Study of Autism Consortium
INO80	Chromatin-remodeling ATPase INO80
iN cell	Induced neuronal cell
INPP4A	Inositol polyphosphate phosphatase 4A
iPSC	Induced pluripotent stem cell
ISWI	Imitation SWI
IUGR	Intrauterine growth restriction

JAK	Janus kinase
JARID1C	Jumonji/ARID domain-containing protein 1C
JMJD	Jumonji C domain-containing histone demethylase
KANSL1	KAT8 regulatory NSL complex subunit 1
KAT	Lysine acetyltransferase
KDM	Lysine demethylase
KMT	Lysine methyltransferase
KMT2A	Lysine (K)-specific methyltransferase 2A
KsRE	Kisspeptin response element
LASS2	Longevity assurance homolog 2 gene
LBs	Lewy bodies
LCLs	Lymphoblastoid cell line
LCM	Laser-capture microdissection
LC-MS	Liquid chromatography/mass spectroscopy
LEARn	Latent early-life associated regulation
LG	Licking and grooming
LGA	Large for gestational age
LIF	Leukemia inhibitory factor
LIMK1	Lim kinase 1
LinDA	Linear DNA amplification
LINE	Long interspersed nuclear/nucleotide element
LLD	Late-life depression
lncRNA	Long non-coding RNA
LOD	Logarithm of the odds
LR	Low responder
LRRK2	Leucine-rich repeat kinase 2
LSD	*d*-Lysergic acid diethylamide
LSD1	Lysine-specific demethylase 1
LTP	Long-term potentiation
LTR	Long terminal repeat
MAGEL2	MAGE-like 2
MALDI-TOF	Matrix-assisted laser desorption/ionization–time-of-flight
MALDI-TOF MS	Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry
MAO	Monoamine oxidase
MAOIs	Monoamine oxidase inhibitors
MAPK	Mitogen-activated protein kinase
MAT2A	Methionine adenosyltransferase II alpha
MB-COMT	Membrane-bound isoform of COMT
MBD	Methyl-CpG-binding domain
MCI	Mild cognitive impairment
mCpG	Methyl-CpG
MDD	Major depressive disorder
MDE	Major depressive episode
MDH	Malate dehydrogenase
MDMA	3,4-Methylenedioxy methamphetamine
me	Methylation
MeA	Medial amygdala

MeCP Methyl-CpG-binding protein
MeCP2 Methyl-CpG-binding protein 2
MeDIP Methylated DNA immunoprecipitation
MeDIP-seq Methylated DNA immunoprecipitation sequencing
MeHg Methylmercury
MELAS Myopathy, encephalopathy, lactic acidosis, stroke-like episodes
MEM Minimal essential medium
MEN1 Multiple endocrine neoplasia
MERRF Myoclonus epilepsy with ragged red fibers
MEST Mesoderm-specific transcript
MET Methionine adenosyltransferase
mGluR Metabotropic glutamate receptor
MGMT Methylguanine DNA methyltransferase
MIH L-Methionine-induced hypermethylation
MIM Mendilian inheritance in man
MIP Methylation-independent PCR
MIRA Methylated CpG island recovery assay
miRNA microRNA
mitosRNA Mitochondrial genome-encoded small RNA
MKRN3 Makorin ring finger protein 3 gene
MLL Mixed-linkage leukemia
MLL1 Mixed-lineage leukemia 1
MLPA Multiplex ligation-dependent probe amplification
MNase Micrococcal nuclease
MOR μ-Opioid receptor
mPFC Medial prefrontal cortex
MPP+ 1-Methyl-4-phenylpyridinium
mQTL Methylation quantitative trait loci
MR Mineralocorticoid receptor
MRD Mental retardation, autosomal dominant
mRNA Messenger RNA
MRXS Mental retardation, X-linked, syndromic
MS Mass spectrometry
MS Maternal separation
MS-HRM Methylation-sensitive high-resolution melting
MSK1 Mitogen- and stress-activated protein kinase 1
MS-MLPA Methylation-specific multiplex ligation-dependent probe amplification
MSN Medium spiny neuron
MSP Methylation-specific PCR
MS-qFRET Methylation-specific quantum dot fluorescence resonance energy transfer
Ms-SNuPE Methylation-sensitive single nucleotide primer extension
MSUS Unpredictable maternal separation and maternal stress
mtDNA Mitochondrial DNA
mtDNMT1 Mitochondrial DNMT1
MTHF Methyltetrahydrofolate
MTHFR Methylenetetrahydrofolate reductase
mTOR Mammalian target of rapamycin

MTR	Methionine synthase
MYST	MOZ, YBF2, SAS2, TIP60
MZ	Monozygotic
NAc	Nucleus accumbens
NAD^+	Nicotinamide adenine dinucleotide
nBAF	Neuronal BAF complex
NCBI	National Center for Biotechnology Information
NCOA6	Nuclear receptor coactivator 6
N-CoR	Nuclear receptor co-repressor
NCOR1	Nuclear receptor co-repressor 1
ncRNA	Non-coding RNA
NCSs	Neural stem cells
ND1	NADH dehydrogenase subunit 1
ND6	NADH dehydrogenase subunit 6
NDDs	Neurodevelopmental disabilities
NDMA	*N*-methyl-D-aspartate
NDN	Necdin gene
nDNA	Nuclear DNA
NDRs	Nucleosome-depleted regions
NEDD4	Neural precursor cell expressed, developmentally downregulated 4, E3 ubiquitin protein ligase
NEP	Neprilysin
NET	Norepinephrine transporter
NF-κB	Nuclear factor κ-light-chain-enhancer of activated B cells
NFT	Neurofibrillary tangle
NGFI-1A	Nerve growth factor-inducible protein 1A
NGFIA	Nerve growth factor-inducible A gene
NGFIA	Nerve growth factor-inducible A transcription factor
NGFI-A	Nerve growth factor-inducible protein A
NGFIB	Nerve growth factor-inducible B
Ngn	Neurogenin
NGS	Next-generation sequencing
NIH	National Institutes of Health
NIPBL	Nipped-B-like protein
NLM	National Library of Medicine
NMDA	*N*-methyl-D-aspartate
NMDAR	*N*-methyl-D-aspartate receptor
NNNS	NICU Network Neurobehavioral Scales
NPAP1	Nuclear pore associated protein 1
NPCs	Neural progenitor/precursor cells
NPS	Non-psychiatric subject
NPY	Neuropeptide Y
NR3C1/Nr3c1	Nuclear receptor subfamily 3, group C, member 1
NRG1	Neuregulin 1 gene
NRSE/RE1	Neuron-restrictive silencer element/RE-1
NRSF	Neuron-restrictive silencer factor
NRSF/REST	Neuron-restrictive silencer factor/RE1-silencing transcription factor

NSC	Neural stem cell
NSD1	Nuclear receptor-binding SET domain protein 1
NSL	Non-specific lethal
NT2 cells	Neuronal precursor 2 cells
NTDs	Neural tube defects
NuRD	Nucleosome remodeling deacetylation
OAT	Ornithine aminotransferase
OATL1	Ornithine aminotransferase-like 1
OPC	Oligodendrocyte precursor
ORF	Open reading frame
OSKM	Oct3/4, *Sox2*, *Klf4*, and c-*Myc*
OVX	Ovariectomized
OxBS-seq	Oxidative bisulfite sequencing
OXPHOS	Oxidative phosphorylation
P4	Progesterone
P10	Postnatal day 10
PAGR1	PAXIP1-associated glutamate-rich protein 1
pancRNAs	Promoter-associated non-coding RNA
PARK2	Parkin
PAXIP1	PAX interacting protein 1
PB	Phenylbutyrate
PBMC	Peripheral blood mononuclear cell
PCAF	p300/CBP-associated factor
PcG	Polycomb group
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PCTK1	Cyclin-dependent kinase 16 gene
PD	Parkinson's disease
PDE4B	Phosphodiesterase 4B, cAMP-specific
PET	Positron emission tomography
PFC	Prefrontal cortex
PGC	Primordial germ cell
PGC	Psychiatric Genetics Consortium
PHD	Plant homeodomain
PHF	PHD finger protein
PI3K	Phosphatidylinositol 3-kinase
PINK1	PTEN-induced putative kinase 1
piRNA	PIWI-interacting RNA
PK	Protein kinase
PLP	Myelin proteolipid protein
PND	Postnatal day
POLG	mtDNA polymerase γ
PolgA	mtDNA polymerase γ catalytic subunit
POMC	Proopiomelanocortin
PP	Phosphatase
PP1	Protein phosphatase 1
PPD	Postpartum depression

PPI	Protein–protein interaction
PRC	Polycomb repressive complex
PRC2	Polycomb repressive complex 2
PR-DUB	Polycomb repressive deubiquitination
PRMT	Protein arginine methyltransferase
PRS	Prenatal restraint stress
PSEN1	Presenilin 1 gene
PSEN2	Presenilin 2 gene
PSY	Psychosis
PTMs	Posttranslational modifications
PTSD	Posttraumatic stress disorder
PVN	Paraventricular nucleus of the hypothalamus
PWS	Prader–Willi syndrome
Q2ChIP	Quick and quantitative ChIP
QD-FRET	Quantum dot FRET
QK1	Quaking homolog, KH domain gene
RA	Retinoic acid
RBBP5	Retinoblastoma binding protein 5
RCOR1/2	REST co-repressor 1 and 2
RE1	Repressor element 1
RELN	reelin
REST	Repressor element 1 silencing transcription factor
Rho kinase 1	ROCK1
RI	Recombinant inbred
Ring1B	Ring finger protein 1B
RIX	Recombinant inbred intercrosses
RMST	Rhabdomyosarcoma 2-associated transcript
RNA-seq	RNA sequencing
RRBS	Reduced representation bisulfite sequencing
rRNA	Ribosomal RNA
RT-PCR	Reverse transcription–polymerase chain reaction
RTS	Rubinstein–Taybi syndrome
RTT	Rett syndrome
S100β	S-100 calcium-binding protein, beta chain
SAH	*S*-adenosylhomocysteine
SAHA	Suberoylanilide hydroxamic acid
SAHH	SAH hydroxylase
SAM	*S*-adenosylmethionine (*S*-adenosyl-L-methionine)
SAT1	Spermidine/spermine N^1-acetyltransferase
Sat2	Satellite 2 DNA sequence
Satb2	Special AT-rich sequence-binding protein
SBBYSS	Say–Barber–Biesecker–Young–Simpson syndrome
SCFAs	Short-chain fatty acids
SEP	Socioeconomic position
SES	Socioeconomic status
SET	Su(var)3-9, Enhancer-of-zeste, Trithorax
SGA	Small for gestational age

SGZ	Subgranular zone
Shh	Sonic hedgehog
SIN3A	SIN3 transcription regulator family member A
siRNA	Small (short) interfering RNA
SIRT/Sirt	Sirtuin
SIRT1	Sirtuin 1 (NAD-dependent deacetylase sirtuin 1)
SKI	V-ski avian sarcoma viral oncogene homolog
SMA	Spinal muscular atrophy
SMAD1	Mothers against decapentaplegic homolog 1
SMARC	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin
SMARCA2	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 2
SMC	Structural maintenance of chromosomes
SMN	Survival motor neuron gene
SMOX	Spermine oxidase gene
SMRT	Single-molecule real-time
SMS	Smith–Magenis syndrome
SMS	Spermine synthase
SMUG1	Single-strand-selective uracil–DNA glycosylase 1
SNARE	Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNCA	alpha-Synuclein gene
snoRNA	Small nucleolar RNA
SNP	Single nucleotide polymorphism
SNRI	Selective noradrenaline reuptake inhibitor
SNRPN	Small nuclear ribonucleoprotein polypeptide
SNURF	SNRPN upstream reading frame
SNVs	Single nucleotide variants
Sox	SRY-box containing gene
SOX10	Sex-determining region Y box containing gene 10
SP	Senile plaque
SRCAP	SNF2-related CBP activator protein
SRSF1	Serine/arginine-rich splicing factor 1 gene
SSRI	Selective serotonin reuptake inhibitor
STAT	Signal transducer and activator of transcription
STAT3	Signal transducer and activator of transcription 3
STG	Superior temporal gyrus
STX1B	Syntaxin 1B gene
SUMO1–4	Small ubiquitin-like modifier 1–4
SVZs	Subventricular zones
SWI/SNF	SWItch/Sucrose NonFermentable
SWR1	SNF2 family helicase Swr1
SZ	Schizophrenia
T	Thymine
T3	Triodoithyronine
TAB-seq	TET-assisted bisulfite sequencing
TALE	Transcriptional activator-like effector
TALEN	Transcription activator-like effector nuclease

TBT	Tributyltin
TDG	Thymine–DNA glycosylase
T-DMR	Tissue-dependent and differentially methylated region
TET	Ten–eleven translocation
TET1	Ten–eleven translocation 1
TF	Transcription factor
TGF-β	Transforming growth factor β
TH	Tyrosine hydroxylase
THC	Tetrahydrocannabinol
THF	Tetrahydrofolate
THRb	Thyroid hormone receptor beta
TMEM59	Transmembrane protein 59 gene
TNFRSF21	Tumor necrosis factor receptor superfamily member 21
TrkB	Tyrosine receptor kinase type B
tRNA	Transfer RNA
TSA	Trichostatin A
TS-DMRs	Tissue-specific differentially methylated regions
TSS	Transcription start site
TSST	Trier Social Stress Test
UBA6	Ubiquitin-like modifier activating enzyme 6
UBE2A	Ubiquitin protein ligase E2A
UBE3A	Ubiquitin protein ligase E3A
UBE3A-AST	UBE3A antisense transcript
UCSC	University of California Santa Cruz
UDG	Uracil–DNA glycosylase
UDP-glucose	Uridine diphosphate glucose
UFL1	UFM1-specific ligase 1
uH2A	Monoubiquityl histone H2A
UL	Upper layer
UPD	Uniparental disomy
US	Unconditioned stimulus
UTR	Untranslated region
UTX	Ubiquitously transcribed tetratricopeptide repeat, X chromosome
VCFS	Velocardiofacial syndrome
VECs	Variably erased CpG islands
VGLUT1	Vesicular glutamate transporter 1
VNTR	Variable number of tandem repeats
VPA	Valproic acid
VSNL1	Visinin-like 1 gene
vSTR	Ventral striatum
VTA	Ventral tegmental area
VZ	Ventricular zone
WDR	WD repeat domain
WES	Whole exome sequencing
WGBS	Whole genome bisulfite sequencing
WGS	Whole genome sequencing
WHO	World Health Organization

Wnt	Wingless-type
WS	Williams syndrome
WSTF	Williams syndrome transcription factor
XLID	X-linked intellectual disability
YB1	Major histocompatibility complex, class II, Y box-binding protein I; DNA-binding protein B
YY1	Yin Yang 1 transcription factor

Glossary

5-Hydroxymethylcytosine (5hmc): An epigenetic modification of DNA that can result from the oxidation of 5-methylcytosine by the TET family of enzymes.

5-Methylcytosine (5mC): An epigenetic modification of DNA that usually occurs at CpG dinucleotides and often correlates with repressed gene expression.

Acute drug use: In humans, often refers to casual but not pathological drug use; in rodents, usually refers to a single exposure to a drug of abuse.

***ad libitum*:** Unlimited access.

Affective disorder: Mental disorder that results in persistent negative changes in mood. In this book it is used as a synonym for mood disorders, including depression and anxiety.

Alpha thalassemia/mental retardation syndrome X-linked (ATRX): A chromatin-remodeling factor and DNA methylation modifier that leads to an X-linked disorder if there is a mutation. Mutations in this gene cause the alpha thalassemia/mental retardation syndrome, which is manifest by intellectual delays, facial abnormalities, urogenital defects, and alpha-thalassemia.

Alzheimer's disease: The most common neurodegenerative disorder and the primary form of dementia in the elderly. Neurodegeneration in Alzheimer's disease leads to memory loss accompanied by changes of behavior and personality severe enough to affect daily life.

Amphetamine, atomoxetine, methylphenidate: Drugs used to treat attention deficit hyperactivity disorder.

Aneuploidic disorders: Characterized by extra or deleted copies of X chromosomes.

Angelman syndrome: Complex genetic disorder that affects the nervous system. Characteristic features of this condition include delayed development, intellectual disability, severe speech impairment, and problems with movement and balance (ataxia). Most affected children also have recurrent seizures (epilepsy), a happy disposition, and microcephaly. Delayed development becomes noticeable by the age of 6–12 months, and other common signs and symptoms usually appear in early childhood.

Anhedonia: Inability of an act or object that normally creates pleasure to elicit that response.

Antidepressant: A drug used to treat major depressive disorder.

Antipsychotic: A drug used to treat psychotic disorders such as schizophrenia.

Apomorphine: A non-selective dopamine agonist.

Arginine vasopressin: Vasopressin that contains arginine, an amino acid, in humans and most other mammals. It is a polypeptide synthesized in the hypothalamus and transported to the posterior pituitary gland from where it is released. Also called antidiuretic hormone.

Astrocytes: Specialized star-shaped glial cells involved in many aspects of brain function.

ATP-dependent chromatin remodeling: The movement or ejection or restructuring of nucleosomes requiring energy from the hydrolysis of adenosine triphosphate (ATP).

Autism spectrum disorders: A range of conditions classified as neurodevelopmental disorders in *Diagnostic and Statistical Manual of Mental Disorders*, 5th edition.

BDNF: BDNF is a secreted protein that is involved in synaptic plasticity and memory formation. BDNF encourages the differentiation of neural stem cells into neurons. In postmitotic neurons, BDNF increases synapses and supports their survival. *BDNF* has eight alternative 5′ non-coding exons and a common 3′ coding exon, which are driven by the individual promoters for spatiotemporal expression.

BiS-seq: The genome-wide sequencing of DNA treated with bisulfite.

Bisulfite conversion: The deamination of non-methylated cytosine bases to uracil by treatment with sodium bisulfite ($NaHSO_3$) while methylated cytosines are resistant to bisulfite conversion.

Bisulfite modification of DNA: The genomic modification of DNA with bisulfite. Bisulfite modification of DNA was originally described as a means of positively identifying 5-methylcytosine residues on individual

strands of DNA. Genomic DNA following bisulfite modification and PCR would convert all Cs to Ts unless a specific position is methylated. The methyl group prevents deamination, leaving the cytosine moiety intact. The conversion of hydroxymethylcytosine to cytosine 5-methylenesulfonate was described some time ago and has been recently revisited. HSO_3 (bisulfite) quickly converts 5-hydroxymethylcytosine to form cytosine-5-methylenesulfonate (CMS), which does not readily undergo deamination.

Bisulfite sequencing: Determining the sequence of bisulfite-converted DNA.

Brain plasticity and synaptic connectivity: Changes in dendritic spine morphology and branching.

Bromodomain: Protein domain that binds acetylated histones.

Causal inference: The process by which investigators determine whether a disease or outcome of interest is a consequence of an exposure that is hypothesized to produce the disease. A key consideration here is whether the putative cause occurs prior to the disease/outcome of interest.

ChIP-on-chip: Combining ChIP and DNA hybridization for genomic microarrays.

ChIP-seq: Combining ChIP and NGS to analyze specific DNA–protein interactions genome wide (Chapter 7).

ChIP-seq: Genome-wide sequencing of material generated following ChIP (Chapter 10).

Chromatin: Histone proteins associated with the cell's DNA that regulate its accessibility to gene transcription machinery. Chromatin comes in two forms: heterochromatin, where the DNA is tightly coiled and therefore inaccessible to the transcriptional machinery, and euchromatin, where the DNA is more loosely associated with histone proteins (Chapter 26).

Chromatin: Nuclear material that makes up the chromosome. It consists of DNA; histones H1, H2A, H2B, H3, and H4 (or variants thereof); and other chromatin-associated proteins (Chapter 21).

Chromatin: The complex of DNA, histones, RNA, and other proteins that comprise the structural basis of chromosomes (Chapter 7).

Chromatin immune precipitation (ChIP): A technique whereby DNA and chromatin proteins are cross-linked with formalin or another agent and sonicated. Antibodies specific to a particular transcription factor or modified histone are used to determine if the pulled-down sequences contain the gene of interest (Chapter 10).

Chromatin immunoprecipitation (ChIP): A method used to identify proteins bound to DNA, and the sequence to which they bind, using an antibody to specifically immunoprecipitate the protein of interest while co-precipitating the bound DNA (Chapter 7).

Chromatin immunoprecipitation assay: A method used to identify the DNA sequence to which proteins such as histones are bound. It uses specific antibodies to immunoprecipitate the proteins.

Chromatin remodeling: The change in the accessibility of DNA by transcription factors brought about by changes in the position of histones and DNA.

Chromatin remodeling complexes: Multimeric protein complexes (often containing numerous subunits) that utilize ATP to alter nucleosome positioning along the DNA template.

Chromodomain: Protein domain that binds methylated histones.

Chromosome conformation capture (3C): Allows mapping of local chromosome interactions through increased frequency of intramolecular ligation between fragments in close proximity.

Chronic drug use: In humans, refers to pathological, compulsive drug use; in rodents, usually consists of at least 7 days of experimenter-delivered drug exposure or extended periods of drug self-administration.

Chronic social defeat stress: Etiologically relevant rodent model of human depression involving chronic exposure to aggression, both physical and sensory. Such stress results in either susceptible (exhibiting symptoms of depression, such as reduced social interaction, anhedonia, increased HPA-axis engagement, etc.) or resilient animals.

Cocaine place preference (CPP): Form of Pavlovian conditioning used to measure motivational effects of objects or experiences.

Coffin–Lowry syndrome: A neurological disease caused by a deficiency in a histone phosphorylase. An X-linked disorder, this syndrome is associated with severe ID and a dysmorphic facial appearance. The most

salient features include a prominent forehead, down-slanting palpebral fissures, orbital hypertelorism, thick lips, a thick nasal septum with anteverted nares, and irregular or missing teeth. Coffin–Lowry syndrome is caused by loss-of-function mutations in the RSK2 gene.

Collaborative Cross (CC): Recombinant inbred lines created by breeding eight founder strains of inbred mice (A/J, C57BL/6J, 129S1/SvlmJ, NOD/LtJ, NZO/HILtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ) chosen to maximize genetic diversity.

Combined bisulfite restriction analysis (COBRA): DNA is subjected to bisulfite conversion and digestion with restriction endonucleases that are specific for sequences containing CpG sites.

Comprehensive high-throughput arrays for relative methylation (CHARM): Uses McrBC (a restriction enzyme that recognizes two closely spaced methylated cytosines) to digest genomic DNA and detect hypermethylated CpG sites using a microarray.

Confounding: Occurs when the effect of an exposure is "mixed together" with the effect of another variable, leading to a biased estimate of association with the outcome (i.e., disease) of interest. Statistical models can control for suspected confounding variables in order to arrive at an adjusted measure that focuses on the uniform (or close to uniform) effect that the exposure has on the outcome of interest.

Corticosteroid: Any one of a number of steroid hormones secreted by the adrenal cortex. In humans, the major corticosteroid is cortisol; in rodents, corticosterone. Mineralocorticoids, such as aldosterone, regulate blood volume and renal function. Glucocorticoids, such as cortisol, regulate immune function and glucose metabolism but are most significantly associated with the stress response.

Corticotropin-releasing hormone: A hypothalamic peptide hormone that stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH) from the anterior pituitary gland.

CpG island: Cytosine–phosphate–guanine dinucleotide-rich region of the genome usually located in and around the promoter region of genes (Chapter 2).

CpG island: Region with a high proportion of cystosine–guanine dinucleotides (Chapter 13).

CpG islands: Regions of DNA enriched for CG dinucleotides. These are typically 300–3000 bp long, located upstream of gene coding regions, and usually protected from DNA methylation (Chapter 7).

Cross-fostering: In rodents, this process involves the removal of pups from their biological mother at birth and placing them with a foster mother. This allows for the examination of maternal care environment and genetic background separately.

Cyclin-dependent kinase-like 5 (CDKL5): Gene that is a member of the Ser/Thr protein kinase family and encodes a phosphorylated protein with protein kinase activity. Mutations in this gene have been associated with X-linked infantile spasm syndrome (ISSX), also known as X-linked West syndrome and Rett syndrome (RTT).

Cytidine: A nucleoside formed by the condensation of cytosine and the sugar ribose.

Cytosine: A pyrimidine base that is one of the fundamental components of DNA and RNA.

Dam: Female parent.

***De novo*:** Addition of a new chemical modification where none existed before.

***De novo* methylation:** A process mediated by DNMT3 methyltransferases that involves the establishment of methylation patterns during embryonic development.

Demethylases: The enzymes that catalyze the removal of a methyl group from 5-methylcytosine and also regulate the chromatin state.

Differentially DNA-methylated region (DMR): A region of DNA that is methylated differentially in the two chromosomes of a diploid cell. These are often associated with genomic imprinting.

DNA methylation: A heritable, reversible epigenetic modification in which a methyl group is covalently added to a DNA sequence, usually the fifth carbon of the cytosine pyrimidine ring in a CpG dinucleotide (i.e., 5mC). Recent research is also revealing the presence of non-CG methylation in mammalian cells (Chapter 7).

DNA methylation: An epigenetic modification that involves the covalent addition of methyl groups to the carbon 5 of the cytosine ring, generally in CpG dinucleotides (Chapter 2).

DNA methylation: The addition of a methyl group (CH_3) to the 5′ position of the cytosine pyrimidine ring, forming 5-methylcytosine (5-mC), a mark that in the promoter region is usually associated with gene silencing (Chapter 17).

DNA methyltransferase (DNMT): A family of enzymes (DNMT1, 3a, and 3b) that catalyze the addition of a methyl group to cytosine bases (Chapter 7).

DNA methyltransferase (DNMT): Enzymes that catalyze the addition of methyl groups to the 5′ carbon positions of cytosine nucleotides within DNA (Chapter 21).

DNA methyltransferase (DNMT): The enzymes that aid the process of addition of a methyl group to position 5 of the cytosine pyrimidine ring. DNMT1 and DNMT3 are the major human DNA methyltransferases (Chapter 2).

DNaseI-seq: A method that distinguishes open chromatin regions based on their hypersensitivity to DNaseI digestion. Sequencing these genomic fragments can generate genome-wide maps of chromatin accessibility.

Dopaminergic: Dopamine neuromodulator-releasing neurons.

Drug addiction: Pathological drug seeking and use in spite of adverse physiological, psychological, and social consequences.

Early-life stress: Early-life maltreatment (e.g., neglect; physical, sexual, or psychological abuse) or stress; often modeled in rodents by preweaning maternal separation or chronic handling.

Effect size: A descriptive statistic that indicates the strength of a relationship between an exposure and outcome; often (but not always) expressed as the difference in mean outcomes of an exposed versus unexposed group divided by the standard deviation of the outcome in the unexposed group.

Encephalization: An evolutionary increase in the complexity or size of the brain, including a shift of function to cortical regions of the brain.

Endophenotype: A term used in genetic epidemiology to parse behavioral symptoms into more stable phenotypes with a clear genetic connection.

Environment: In the context of this book, environment is used to refer to exposures that individuals encounter during their lifetime beginning *in utero*. We use this term in the broadest sense to include hormonal, social, stressful, toxicological, and nutritional events.

Epigenetic change: Heritable changes in the DNA that do not alter the nucleotide sequence but can regulate the expression of genes.

Epigenetic mark: A modifying moiety that carries an epigenetic signal (e.g., DNA methylation, methylation and acetylation of histones).

Epigenome: All of the epigenetic marks present throughout the genome of a cell (Chapter 7).

Epigenome: The collection of all DNA methylation variations and/or all histone variants across the genome (Chapter 13).

Epigenome: The overall epigenetic state of a cell that serves as an interface between the environment and the genome (Chapter 26).

Epimutation: Heritable defect involving epigenetic mechanisms of gene expression.

Euchromatin: The less dense form of chromatin that is rich in genes and is thought to be more suitable for active transcription than highly condensed heterochromatin.

Euchromatin: Transcriptionally active chromatin that is less condensed (Chapter 21).

Euchromatin: The loosened state of chromatin that is favorable for transcription. It is generally associated with histone acetylation and non-methylated DNA (Chapter 17).

F (filial): A generation in a breeding experiment that is successive to a parental generation.

Formaldehyde-assisted isolation of regulatory elements (FAIRE): Exploits the solubility of open chromatin in the aqueous phase during phenol:chloroform extraction to generate genome-wide maps of soluble chromatin.

Fragile X syndrome (FXS): Most common inherited cause of ID. Boys and girls with FXS have a phenotype that includes a characteristic physical appearance, cognitive skill deficits, and impaired adaptive behaviors. Many affected children satisfy the criteria for the diagnosis of autism. The prevalence of FXS for males with the full mutation is about 1:3600. Prevalence of the full mutation in females is estimated to be at least 1:4000–1:6000. FXS arises from an expansion of the number of cytosine–guanine–guanine (CGG) triplet repeats occurring within the initial 5′ translated region of the *FMR1* gene (fragile X mental retardation protein gene).

GABAergic: Neurons using γ-Aminobutyric acid (GABA) as neurotransmitter.

GAD67–GFP transgenic mice: Transgenic mice that can be useful for fluorescent labeling of neurons and the study of neurons in the hippocampus.

Gene bodies: Gene bodies are located downstream (3′) to transcription start sites, containing both exons and introns, and extending through the 3′ transcription termination point.

Gene promoter: Region of DNA that initiates transcription of a gene.

Genetic association: A statistical approach to identifying loci that increase the risk for disease. It tests whether the allele frequencies of a polymorphic marker are different in cases than in controls. A positive result is assumed to mean that the allele more frequent in cases is increasing the risk for disease or is tagging another variant allele that does. Today, it is most often applied at the genome-wide level, involving millions of markers and requiring very robust statistical significance to avoid type 1 errors.

Genomic imprinting: An epigenetic phenomenon by which certain genes can be expressed in a parent-of-origin-specific manner.

Germ cell: Reproductive cell in the body; may be a sperm or ovum.

Germ line: The cellular lineage from which eggs and sperm are derived.

GLIB-seq: Genome-wide sequencing method for detecting hydroxymethylcytosine. The technique uses T4 β-glucosyltransferase (BGT) and uridine diphosphate glucose (UDP-glucose) to add a glucosyl group to protect hydroxymethylcytosines in genomic DNA. Sodium periodate is used to oxidize glycol groups to form aldehydes, which are then biotinylated and precipitated with streptavidin beads.

GLP, G9a: Key histone lysine methyltransferases for H3K9me1 and H3K9me2.

Glucocorticoid: A steroid hormone that is central to the stress response.

Glutaminergic: Glutamate (excitatory) neurotransmitter-releasing neurons.

Heritability: The portion of the variance of a trait in a population that can be attributed to genetics. Narrow-sense heritability, h^2, is the portion of the variance that is explained by additive genetic effects; broad-sense heritability, H^2, is the fraction explained by all genetic effects including interactions.

Heterochromatin: Dense chromatin that is thought to be less transcriptionally active than euchromatin. Heterochromatin can be defined in two subtypes. Constitutive heterochromatin includes structural regions of the chromosome, such as centromeres and telomeres, whereas facultative heterochromatin is involved in silencing of specific genes in a given cell type (Chapter 18).

Heterochromatin: The condensed state of chromatin not accessible to transcription factors. It is generally associated with methylated DNA regions bound by histone deacetylases (Chapter 2).

Heterochromatin: Transcriptionally silent chromatin that is highly compact (Chapter 21).

Heteroplasmy: The presence of a mixture of more than one type of mtDNA within a cell. Mutations usually affect some but not all mtDNA molecules within a cell; ultimately, the proportion of normal and mutated mtDNA in different tissues determines the clinical pathology. It is possible that heteroplasmy exists with respect to epigenetic modifications of mtDNA.

High-performance liquid chromatography (HPLC): A chromatographic technology used to separate and quantitate mixtures of substances in solution.

High-resolution melting (HRM) analysis: A real-time, PCR-based method that relies on the rate of DNA melting reflecting differences in the C:T content determined by the level of methylation.

Histone: Chromosomal architectural proteins that bind DNA within nucleosomes. In eukaryotes there are four core histones (H2A, H2B, H3, and H4), the non-nucleosomal linker histone H1, and variant histones.

Histone acetyltransferase: An enzyme that catalyzes the acetylation of histones (Chapter 25).

Histone acetyltransferases: The enzymes that catalyze the addition of acetyl groups to specific lysine residues in the histone tails (Chapter 2).

Histone code: The hypothesis that the locations and types of histone modifications, through chromatin remodeling and/or recruitment of transcription factors, predict the effects of those modifications on gene expression (Chapter 7).

Histone code: The specific pattern of histone protein modifications that delineate the parts of the genome to be expressed at a given point in time in a given cell type (Chapter 26).

Histone deacetylase: An enzyme that catalyzes the deacetylation of histones (Chapter 25).

Histone deacetylase: The enzymes that remove the acetyl group from specific lysine residues of the histone tail (Chapter 2).

Histone deacetylase inhibitor: A drug that inhibits the enzyme histone deacetylase.

Histone lysine mono-, di-, and trimethylation: Histone lysine methylation is catalyzed by a family of proteins that contain a SET domain. Histone lysine mono-, di-, or trimethylation is performed by the addition of one, two, or three methyl groups ($–CH_3$) to the nitrogen of the lysine amine group ($–NH_3^+$), respectively.

Histone modification: Posttranslational modification of histones that regulates the chromatin structure. The modifications include acetylation, methylation, and phosphorylation, to name a few (Chapter 2).

Histone modifications: Covalent posttranslational modifications of amino-acidic residues on the histone tails, such as acetylation, methylation, phosphorylation, ubiquitylation, and sumoylation. The combination of those modifications, and their interplay with DNA methylation and chromatin remodeling proteins, regulates the chromatin structure in a dynamic fashion (Chapter 17).

hMeDIP: An immunopurification (5-hmC antibody) strategy to enrich sequences containing hydroxymethyl- CpG.

hMeDIP-seq: A process where sonicated DNA fragments are precipitated and the enriched sequences are purified and sequenced using NGS.

HPA axis: The neuroendocrine stress axis, comprised of the PVN of the hypothalamus, anterior pituitary corticotrophs, and adrenal cortex.

HpaII tiny fragment enrichment by ligation-mediated PCR (HELP): Methylation-sensitive restriction digested DNA fragments are labeled with fluorescent dyes and co-hybridized to an oligonucleotide microarray.

Huntington's disease: Progressive neurodegenerative disorder resulting in cognitive impairment, choreiform movements, and psychiatric and behavioral disturbances.

Hypothalamic–pituitary–adrenal axis: A set of interactions among the hypothalamic region of the brain with the pituitary and adrenal glands that is mediated by hormones released in response to stress.

Illumina® Infinium® HumanMethylation27 BeadChip™ array: An earlier release of the Illumina Infinium methylation product line. It offers over 27,000 single site CpG probes corresponding to many of the highly regulated CpG sites in the human genome.

Illumina® Infinium® HumanMethylation450 BeadChip™ array: Second-generation BeadChip produced by Illumina that contains over 480,000 probes, targeting over 99% of genes and 96% of CpG islands. Differences of 20% are detectable with 99% confidence.

Imprinted gene: Gene in which one copy is epigenetically silenced.

Imprinting: Epigenetic regulation in which maternally and paternally inherited alleles are differentially expressed owing to *cis*-acting modifications of DNA or histones inherited from parental chromosomes.

Imprinting center: A chromosomal region that is required for genomic imprinting of a given imprinted region. If the imprinting center is missing or altered, genes in that region will not undergo normal imprinting.

***In silico*:** Performed on computer or by computer simulation.

***In vitro*:** Outside the body.

***In vivo*:** Inside the body.

Intellectual disability: A disability characterized by significant limitations both in intellectual functioning (reasoning, learning, problem solving) and in adaptive behavior, which covers a range of everyday social and practical skills. This disability originates before the age of 18.

Intrauterine growth restriction (IUGR): Inadequate growth of the fetus *in utero* that can result from maternal nutritional deficiencies or insufficient oxygen supply, among other causes.

iPSC: Induced pluripotent stem cells, called iPSCs, are pluripotent stem cells artificially derived from somatic cells by forced expression of specific transcription factors, such as *Oct3/4*, *Sox2*, c-*Myc*, and *Klf4*. Similarly to embryonic stem cells (ESCs), iPSCs have potential to self-renew and develop into any type of cells. The methods for reprogramming to iPSCs from mouse and human cells were established by Yamanaka and colleagues in 2006 and in 2007, respectively. Since this landmark study, many researchers have also established various methods for reprogramming that bypass iPSC generation and direct the production of various cell types by forced expression of a specific set of transcription factors (direct reprogramming).

Linkage analysis: An analytical process that explores whether or not two variant sequences segregate independently in pedigrees. When one of the variants is an assumed unobserved disease locus then linkage is said to be between DNA markers and a disease. The relationship between the unobserved genotype at the disease locus and the phenotype is the linkage model. In non-parametric linkage, a model does not need to be specified and the analysis is exploring whether in pairs of relatives ascertained to be concordant for the disease there is on average greater sharing of alleles identical by descent than expected for the given relationship at any location in the genome.

L-Methionine: An essential amino acid that is the metabolic precursor of *S*-adenosylmethionine.

Long ncRNAs (lncRNA): The RNA transcripts that are more than 200 nucleotides long and believed to bind to chromatin and recruit other chromatin-modifying complexes (Chapter 2).

Long non-coding RNA (lncRNA): Non-protein coding RNA transcripts longer than 200 nucleotides that may facilitate multiprotein complex docking on DNA (Chapter 21).

Longitudinal study: A study that involves sampling participants at two or more time points, ideally before and after disease onset.

Long-term potentiation (LTP): Lasting enhancement of signal transmission between neurons facilitating learning and memory (Chapter 5).

LTP: Long-term potentiation (LTP) is a long-lasting enhancement in signal transmission between two neurons. LTP has two distinct phases: early phase (E-LTP; lasting 1 to approximately 3 hours) and late phase (L-LTP; lasting at least 24 hours). E-LTP is followed by L-LTP. Whereas E-LTP is independent of protein synthesis, L-LTP requires mRNA and protein synthesis in the postsynaptic cell. LTP is induced by high-frequency stimulation of the neuron leading to elevation of Ca^{2+} in postsynaptic dendritic spines and a prolonged enhancement in signal transmission across the synaptic cleft. In this process, protein kinases, such as CaMK-II and ERK, are activated, followed by changes in gene expression and protein synthesis (Chapter 4).

Maintenance: Replication of an existing chemical modification (e.g., DNA methylation).

Major psychosis: A type of psychiatric disorder that mainly includes schizophrenia and bipolar disorder.

MBD-seq: Genome-wide sequencing method that uses the methyl-binding domain of MBD2 to enrich for CpG sequences bound by this domain (Chapter 10).

MBD-seq: Identification of methylated DNA by precipitation with a methylated DNA-binding protein specific for 5mC followed by detection of precipitated DNA (Chapter 7).

MeDIP: An immunopurification step that enriches for sequences based on the presence of a methylcytosine base.

MeDIP-seq: Genome-wide sequencing of DNA fragments that have been immune precipitated using MeDIP.

Methyl C-seq: Also called BiS-seq, this process is used for shotgun sequencing of BiS converted genomic DNA.

Methylated CpG island recovery assay (MIRA): Using the MBD2/MBD3L1 complex (a methylated DNA-binding protein complex) to enrich methylated DNA fragments for subsequent analysis.

Methylated DNA immunoprecipitation (methyl-DIP or MeDIP): Identification of methylated DNA by precipitation with an antibody specific for 5mC followed by detection of precipitated DNA.

Methylation-dependent restriction enzyme: A restriction enzyme that requires DNA methylation for cleavage.

Methylation-sensitive PCR (MSP): Determination of the methylation status of specific DNA sequences by PCR amplification of a converted template with different primer sets that distinguish methylated DNA and non-methylated (C > T converted) DNA.

Methylation-sensitive restriction enzyme: A restriction enzyme whose activity is blocked or impaired by DNA methylation.

Methylation-sensitive single-nucleotide primer extension (Ms-SNuPE): Determining methylation status of a targeted base bisulfite conversion by primer extension with labeled dCTP or dTTP to distinguish methylated and non-methylated DNA.

Methyl-CpG-binding protein: Proteins containing specific binding domains that allow for direct interactions with methylated DNA.

Methyl-CpG-binding protein 2 (MeCP2): Protein with methyl-binding domains that binds to methylated CpG islands and may recruit chromatin remodeling complexes and influence acetylation of nearby histones.

MethylC-seq: Identification of methylated DNA by shotgun sequencing of bisulfite-converted DNA.

microRNA: A non-coding RNA belonging to the short non-coding RNA category; it is 21–23 nucleotides (nt) in length (Chapter 12).

microRNA: Non-coding RNAs that are 22–23 nucleotides long; they bind to the 3′ untranslated region of specific mRNAs and regulate the expression of certain genes (Chapter 2).

microRNA: Non-protein-coding RNA transcripts that are approximately 22 nucleotides in length that base pair with mRNA transcripts to regulate protein expression (Chapter 21).

Mitochondrion: Eukaryotic organelle with its own mtDNA. Mitochondria are primarily involved in energy (e.g., ATP) production but are also critical for a number of other physiological processes. The word "mitochondrion" originates from the Greek *mitos* ("thread-like") and *khondrion*, diminutive of *khondros* ("granule/grain").

MNase-seq: Distinguishes nucleosome positioning based on the ability of nucleosomes to protect associated DNA from digestion by micrococcal nuclease. Protected fragments are sequenced to produce genome-wide maps of nucleosome localization.

Monoamine oxidase: An enzyme that catalyzes the metabolism of monoamines.

Monotremes: Egg-laying mammals.

mtDNA: Mitochondrial DNA, which in many animal species is small (16,295–16,826 bp), circular, intron-free, double-stranded DNA that contains only 37 genes, of which 13 encode polypeptides/proteins. In contrast to nDNA, all cellular mtDNA is exclusively maternally inherited. Each mitochondrion typically contains multiple mtDNA copies (up to 10) and each cell contains multiple mitochondria (up to thousands).

mtDNA haplotypes: In the course of human migrations, people have accumulated distinctive variations from the mtDNA of the ancestral "mitochondrial Eve," resulting in haplotypes characteristic of different ethnic groups. It appears that these haplotypes can modulate global DNA methylation and might be associated with susceptibility factors in psychiatric disorders. It is possible that various haplotypes may differentially influence the susceptibility of mtDNA for epigenetic modifications.

Myelination: Myelination is one of the characteristics observed during oligodendrocyte maturation. In culture, oligodendrocytes develop from oligodendrocyte precursors (OPCs) by extending processes and forming sheet-like myelin protein-containing protrusions. In the presence of neuronal axons, OPCs extend their processes, and upon contact with the axon initiate a wrapping process, forming the compact myelin sheath.

Myelodysplastic syndromes: Hematological conditions where stem cells of bone marrow do not mature into healthy blood cells.

Neurodevelopmental disabilities: Chronic conditions that affect the developing and the mature nervous system such as cerebral palsy, mental retardation, behavioral syndromes, or neurologic conditions.

Neuropsychiatric disorder: A disorder due to dysfunction of the brain spanning the medical specialties of neurology as well as psychiatry.

Neurotrophic: Having a trophic effect on neurons, including changes in spinogenesis, dendritic architecture, axonal growth, and neurogenesis. A number of neurotrophic factors have been implicated in psychiatric disease, including brain-derived neurotrophic factor (BDNF) and fibroblast growth factor 2 (FGF2).

Neurotrophic factor: A family of proteins that induce the survival, development, and function of neurons.

Next-generation sequencing (NGS): Highly parallelized DNA-sequencing technologies that produce up to millions of short reads (25–500 bp) at a relatively low cost and in a short time. By contrast, traditional Sanger sequencing produces only a few hundred reads per run (Chapter 7).

Next-generation sequencing (NGS): Refers to any number of massively parallel deep sequencing methods that generate short stretches of DNA sequence (shotgun libraries) that are then aligned to reference genomes or assembled using relatively high-speed computing (Chapter 10).

Non-coding RNA (ncRNA): Any of a number of RNAs that do not code for a protein. These include small interfering RNA (siRNA) and microRNA (miRNA), which are actively involved in the regulation of messenger RNA (mRNA) and protein expression, as well as a growing number of more exotic species (Chapter 16).

Non-coding RNA (ncRNA): RNA molecules that are not translated into proteins. They can epigenetically modify a variety of cellular processes. Some non-coding RNAs important in epigenetics are microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and large non-coding RNAs (lncRNAs) (Chapter 2).

Non-coding RNA (ncRNA): RNAs that are transcribed from DNA but are not translated into protein (Chapter 12).

Non-organic psychiatric disorder: A psychiatric disorder not due to any known underlying brain dysfunction.

Nucleosome: The basic unit of transcription; 147 bp of DNA wrapped ~1.7 times around an octamer of histones (two copies each of H2A, H2B, H3, and H4) (Chapter 21).

Nucleosome: The repeating unit of chromatin structure; one nucleosome is comprised of 147 bp of DNA wrapped around a protein octamer including two molecules each of the core histones H2A, H2B, H3, and H4 (Chapter 7).

Oligodendrocytes: Specialized glial cells that wrap around axons.

One-carbon metabolism: A metabolic pathway in the body centered around folic acid.

Organic psychiatric disorder: A psychiatric disorder due to transient or permanent brain dysfunction due to specific organic factors such as aging, trauma, or tumors.

OxBiS-seq: A chemical method (potassium perruthenate, $KRuO_4$) to oxidize 5-hmC to 5-fC. Following BiS conversion, the 5-fC is converted to T while the original 5-mC remains a C. The technique can be used both genome wide and at specific loci.

Parkinson's disease: The second most common neurodegenerative disorder after Alzheimer's disease, clinically characterized by resting tremor, rigidity, bradykinesia, and postural instability as well as non-motor symptoms such as autonomic insufficiency, cognitive impairment, and sleep disorders.

Perinatal period: The period including both gestation and the postpartum period.

Phelan–McDermid syndrome: Phelan–McDermid syndrome is a more recently recognized disorder featuring ID, significant language impairment, poor social communication, hypotonia, and dysmorphic facies. It is due to a microdeletion on chromosome 22q13 which leads to haploinsufficiency of the SHANK3 gene.

Phenotype: Any observable characteristic of an organism, including its behavior. An organism's phenotype is a product of its genetics and its environment (Chapter 26).

Phenotype: Observable characteristics or traits of an organism (Chapter 9).

Polyamines: Compounds containing more than one amino group, such as spermine and spermidine.

Postnatal (postpartum) period: In humans this usually refers to the first year after birth and the first 3 weeks in rodents.

Posttraumatic stress disorder: A psychiatric condition that arises in response to experiences such as combat or assault and is characterized by hyperarousal, flashbacks, and avoidance.

Prader–Willi syndrome: Complex genetic condition that affects many parts of the body. In infancy, this condition is characterized by weak muscle tone (hypotonia), feeding difficulties, poor growth, and delayed development.

Prenatal period: Time *in utero* or prior to birth.

Primary epimutation: A heritable change in gene expression that is not associated with any change to the DNA sequence (Chapter 18).

Primary epimutation: Also referred to as true epimutation, it does not involve a change in the DNA sequence; more common than secondary epimutation (Chapter 27).

Progenitor cell: Progenitor cells are any dividing cells with the capacity to differentiate. In contrast to stem cells, progenitor cells include putative stem cells in which self-renewal has not yet been demonstrated.

Psychopathology: The manifestation of mental illness in the form of phenotype, including abnormal behavior and physiology.

Psychopharmacology: The study of drugs used to treat psychiatric disorders.

Psychostimulants: Psychoactive drugs such as cocaine, amphetamine, or methylphenidate that stimulate the central nervous system by boosting the release of specific neurotransmitters and neuromodulators (e.g., dopamine).

Psychotropic drug: A drug used to treat psychiatric disorders.

Pyrosequencing: A sequencing-by-synthesis method used to quantitatively determine the methylation of individual CG cytosines over short stretches of DNA (from 50 to 120 bp).

Rapamycin (mTOR) signaling: A protein kinase that controls cell growth, proliferation, and survival.

Rasopathies: Clinically defined group of medical genetic syndromes caused by germ line mutations in genes that encode components or regulators of the Ras/mitogen-activated protein kinase (MAPK) pathway. These disorders include neurofibromatosis type 1, Noonan syndrome, Noonan syndrome with multiple lentigines, capillary malformation–arteriovenous malformation syndrome, Costello syndrome, cardiofaciocutaneous syndrome, and Legius syndrome.

Reduced representation bisulfite sequencing (RRBS): Sequencing of untreated and treated-DNA fragments that were adapter ligated and size selected after restriction enzyme digestion (Chapter 7).

Reduced representation bisulfite sequencing (RRBS-seq): A technique that first digests genomic DNA with restriction enzymes to enrich in CG-rich regions. Following gel purification, these DNA fragments are treated with BiS and sequenced. The technique is applicable to genome-wide sequencing following library preparation (Chapter 10).

Retrospective case-control study: A study that involves selecting participants from unrelated individuals with known diagnoses (i.e., cases), as well as a comparable set of controls, with both sets of participants drawn from the same source population.

Rett syndrome/MeCP2: A neurodevelopmenal disorder that affects girls almost exclusively. It is characterized by normal early growth and development followed by a slowing of development, loss of purposeful use of the hands, distinctive hand movements, slowed brain and head growth, problems with walking, seizures, and intellectual disability.

Reverse causation: Occurs when the exposure–disease association is actually a consequence (rather than a cause) of the disease itself; for example, in a sample of individuals with and without a given disease, any observed disease-associated epigenetic variation (i.e., the exposure) could be a result of having the disease itself.

Ribosomal RNA: The RNA component of the ribosome essential for protein synthesis in all living organisms.

Rubinstein–Taybi syndrome: Syndrome associated with the dysfunction of a histone acetyltransferase. An autosomal dominant disorder, it is characterized by ID and physical anomalies, most notably broad radially

deviated thumbs and phalluses and a postnatal growth deficiency that is later followed by excessive weight gain. There are also characteristic dysmorphic facial features and an increased risk for developing tumors. Rubinstein–Taybi syndrome is caused by mutations in the cAMP response element-binding (CREB) protein (CBP) gene.

Secondary epimutation: Heritable defect in gene expression due to a change in the DNA sequence of a gene encoding a protein involved in the epigenetic machinery of a cell.

Self-administration paradigm (rodents): Etiologically relevant model of human drug addiction, where animals undergo operant conditioning to receive intravenous drug injections. Many variations of this behavioral paradigm exist.

Sequenom MassARRAY: High-throughput assay for evaluating methylation of bisulfite-treated DNA. It uses base-specific cleavage and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) introduced previously for SNP discovery.

Setdb1: A H3K9 histone methyltransferase.

Sire: Male parent of a quadruped (Chapter 28).

Sire: Male parent (Chapter 8).

SiRNA: Double-stranded RNA molecules that are 21 nucleotides long and bind to specific mRNA molecules, often resulting in their degradation.

Small for gestational age (SGA): Babies that are born smaller than normal for their gestational age, usually a body weight that is 10% lower than average for their age.

Smith–Magenis syndrome: Disorder manifest by multiple congenital anomalies and ID. The clinical phenotype includes rather distinctive craniofacial and skeletal features that change with age, a history of infantile hypotonia, significant expressive language delay, ID, stereotypies, behavioral problems, and a sleep disorder because of an abnormal circadian secretion pattern of melatonin synaptopathies.

Sodium valproate/valproic acid: A medication used to treat epilepsy and bipolar disorder that is known to have inhibitory effects on histone deacetylase.

Sonication: The process of dispersing, disrupting, or inactivating particles in a sample using sound-wave energy.

Spinogenesis: The production of new dendritic spines, a common form of neural plasticity.

Stem cell: Stem cells are defined as cells with the ability to self-renew and to give rise to daughter cells that have more specialized properties with limited differentiation capacities.

Synaptic plasticity: A change in the efficacy of signaling (a strengthening or weakening) between neurons (Chapter 5).

Synaptic plasticity: Synaptic plasticity is the ability of synapses to change the structure of the synaptic connections. In response to various signals, the number of receptors located on a synapse increases or decreases. Long-term depression (LTD) and long-term potentiation (LTP) are two of the most-studied forms of synaptic plasticity (Chapter 4).

TET proteins: Ten–eleven translocation 1–3 (TET1–3) proteins catalyze the conversion of 5-methylcytosine into 5-hydroxymethylcytosine.

TET1-assisted bisulfite sequencing (TAB-seq): 5-hmC groups are protected with B-GT and UDP-glucose forming 5-gmC. 5-mCs are then oxidized with recombinant mouse TET1 forming 5-caC. Following bisulfite modification, the protected 5-gmC groups remain as Cs while the 5caCs become Ts. The procedure can be used both genome wide and at specific loci.

Transgenerational epigenetic transmission: Transmission of acquired behavioral responses/susceptibilities across generations without any change in the coding sequence of genes/DNA.

Transposon: A common element in mammalian (and other) genomes that is capable of transposing itself (moving) around the genome. Recent research has established that transposons are not transcriptionally silent as once thought, but are actively transcribed and may play a role in both normal physiology and disease. These include the human LINE-1 and Alu elements.

Trier Social Stress Test: A laboratory procedure used to induce stress in research participants.

***UBE3A* gene:** Gene that encodes ubiquitin protein ligase E3A. This enzyme is involved in targeting proteins for degradation within cells. Both copies of the *UBE3A* gene are active in most of the body's tissues, but in the brain only the maternal copy is normally active. If this copy is mutated or lost, it affects many developmental processes including Angelman syndrome.

Uniparental disomy: Inheritance of two copies of a chromosome, or of part of a chromosome, from one parent and no copies from the other parent.

Velocardiofacial syndrome: Autosomal dominant condition caused by a 3-Mb deletion of contiguous genes on chromosome 22q11.2. Multiple organ systems are affected, including the face, palate, and heart.

Viviparity: Giving birth to offspring that developed within the mother.

Williams syndrome: Microdeletion syndrome characterized by a specific phenotype consisting of cognitive impairment in association with a characteristic cognitive profile, unique personality characteristics, distinctive facial features, and cardiovascular disease. In addition, a range of connective tissue abnormalities and multiorgan anomalies is observed. Hypercalcemia and hypercalciuria may be present in infancy.

X inactivation: The epigenetically mediated inactivation of one of the X chromosomes in females often due to formation of a heterochromatin state.

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